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Low temperature scanning electron microscopy of dog and guinea-pig hyaline articular cartilage

D. L. GARDNER*, P. O'CONNOR* AND K. OATES†

**Department of Histopathology, University of Manchester; and University Hospital of South Manchester, Withington, Manchester, U.K. and †Department of Biological Sciences, University of Lancaster, Lancaster, U.K.*

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INTRODUCTION

This paper describes the low temperature scanning electron microscopy (SEM) of the surfaces of unfixed, fully hydrated mammalian articular cartilage. The problems that attend the microscopic examination of biological materials at low temperature have been extensively discussed (Echlin, Ralph & Weibel, 1978) but cartilage surfaces have, so far, attracted little attention.

The three dimensional appearances of articular cartilage have been recorded by reflected light microscopy (RLM) (Gardner & Woodward, 1969), by reflected light interference microscopy (RLIM) (Longmore & Gardner, 1975; 1978) and by conventional SEM (McCall, 1968; Gardner & Woodward, 1969; Inoue, Kodama & Fujita, 1969; Longfield, Dowson, Walker & Wright, 1969; Redler & Zimny, 1970; Clarke, 1971*a*, *b*; Ghadially, Ghadially, Oryschak & Yong, 1976). Although RLM or RLIM may be employed to observe intact, unfixed cartilage, methods dependent on a source of visible light are limited in resolution and depth of focus. These disadvantages can be greatly reduced by SEM, but investigations by this technique have so far been largely restricted to the survey of fixed, dehydrated tissue.

The mechanical properties of articular cartilage are very closely related to the water retained within the domains of the matrix proteoglycans; in turn, it is believed that the shape assumed by these molecules is strongly influenced by the water content of the tissue. The artefactual removal of water from cartilage is likely to cause large changes in proteoglycan configuration and possibly in collagen–proteoglycan relationships. Consequently, the fine structure of hyaline cartilage is subject to gross distortion during the fixation and dehydration necessary for conventional SEM. To avoid these alterations was a main aim of the low temperature techniques adopted in the present investigation.

In these first studies, low temperature procedures were applied to the SEM survey of non-fixed, fully hydrated dog and guinea-pig cartilage (Gardner, Oates & O'Connor, 1979; Gardner, O'Connor & Oates, 1979). One aim of these experiments was to confirm or refute the presence of the secondary (2^{ry}) and tertiary (3^{ry}) surface features of mammalian articular cartilage described in earlier reports (Gardner & McGillivray, 1971). A further purpose was to study the possibility of surveying non-coated material. It was also planned to determine the extent to which the detachment of cartilage from the underlying bone influences 2^{ry} and 3^{ry} surface structure and to assess whether low temperature techniques introduce previously unrecognised artefacts.

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MATERIALS AND METHODS

Fifty seven blocks of hyaline articular cartilage were cut expeditiously from the cartilage of the central part of the lateral and medial femoral condyles of twenty normal male and female young mature beagle dogs of an inbred strain. The time between the intravenous injection of the narcotic used to kill the animals, and the freezing of the blocks was ~ 3 minutes. For comparison with the dog material, blocks of whole distal femur were taken from five 370 g female guinea-pigs immediately after killing. Twenty of the dog femoral condyles were washed with a fine jet of 0·9% sodium chloride before blocks were prepared; 37 condyles were sampled without washing. The guinea-pig femora, with the cartilage of the lateral and medial condyles resting untouched upon the bone of the femoral shafts, remained unwashed.

Each block was adhered to a specially designed stub and immediately quenched in slushy nitrogen at −210° (63 K). The blocks were stored under liquid nitrogen before transport to a modified JEOL JSM 50A SEM. The SEM has an integral front chamber in which frozen tissue can be coated, fractured or otherwise manipulated. Transfer of the tissue from storage to the cold pedestal in the front chamber of the microscope was achieved by means of a transfer tube (Taylor & Burgess, 1977) and a specimen cap. The cold pedestal was maintained at −182° (91 K). At no time after freezing was the tissue exposed to the air.

Once in the antechamber of the SEM, a coating of ~25 nm of gold and, less often, of aluminium was applied to specimen surfaces by high vacuum evaporation. Two of the guinea-pig samples were subjected to preliminary examination in the uncoated state. Individual specimens were then moved forward to a low temperature stage (Fig. 1) maintained at −194° (79 K) by liquid nitrogen fed directly to the stage through flexible, stainless steel tubes. Specimens were examined at accelerating voltages of 10 kV or 20 kV for uncoated or coated specimens respectively, using a beam current of 0·1–0·4 nA.

The rate of loss of water from specimens during coating, and later, during examination, has been considered carefully. Water sublimes very slowly at temperatures below −130° (143 K) even under high vacuum. During studies of frozen hydrated tissue sections, with a much higher surface area/volume ratio than the blocks used in the present investigations, others have consequently believed that "in practice, at specimen temperatures below −130° (143 K), there is unlikely to be significant water loss through sublimation. . . ." (Saubermann & Echlin, 1975). These conclusions appear to be tenable for the SEM viewing of cartilage blocks *in vacuo*. Within certain limits, the lower the temperature, the slower the rate of sublimation. To substantiate this belief, temperature measurements have been made at the specimen surface during coating, and during prolonged examination, under vacuum.

In the present investigations, the beam current used to observe the specimen has not exceeded 0·4 nA at 20 kV used for periods of $\not> 60$ s, scanning areas of $< 1\ \mu m^2$. This is equivalent to the application of $\not> 48\ kJ/cm^2$. When a $< 1\ \mu m^2$ area of frozen tissue is irradiated, calculations show that the heat is conducted away so rapidly that the temperature rise is less than 2°.

The thermal conductivity of ice, K_t, at temperature t is given by the equation (International Critical Tables):

$$K_t = C(1+\alpha t\ 10^{-4}) \times 10^{-4}\ \text{watts/cm}^2/^\circ,$$

where $C = 209$; $\alpha = 17$.

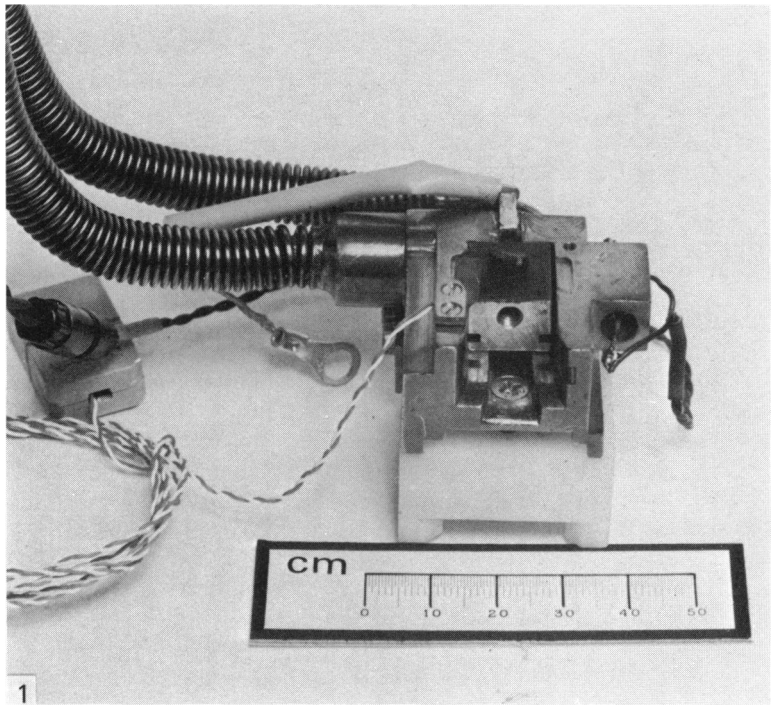


Fig. 1. Specimen stage removed from SEM to show modifications for low temperature electron microscopy. Convoluted, flexible stainless steel tubes pipe liquid nitrogen to stage.

At 0° $K_t = 0{\cdot}0209$ watts/cm²/°.

At $-170°$ $K_t = 0{\cdot}0149$ watts/cm²/°.

When heat is applied, at the rate q watts/s, to a sphere radius r_1 in the centre of a sphere radius r_2 of thermal conductivity K_t, the temperature difference across the conducting thickness $r_2 - r_1$ is given by:

$$\delta T = \frac{q(r_2 - r_1)}{4\pi K_t r_1 r_2}.$$

If

$$r_2 >> r_1, \quad \delta T = \frac{q}{4\pi K_t r_1}.$$

If heat were applied to a hemisphere embedded in an infinite conducting medium, neglecting edge effects, the elevation of the surface temperature would be:

$$\frac{q}{2\pi K_t r_1}.$$

If all the energy of an incident electron beam of 0·4 nA at 20 kV were converted into heat, the energy supplied

$$= 0{\cdot}4 \times 10^{-9} \times 20 \times 10^3 = 8 \times 10^{-6} \text{ watts/s}.$$

Therefore, if all the incident energy falling on a hemisphere 0·5 μm in diameter is lost by conduction into the ice mass, the temperature, after equilibration is reached, would be given by

$$\frac{8 \times 10^{-6}}{2 \times 0{\cdot}0149 \times 0{\cdot}5 \times 10^{-4}} = 1{\cdot}7°.$$ (W. T. W. Potts, personal communication.)

Table 1. *Rates of water loss from ice and from supercooled water* in vacuo: *from Egerton*, 1929, *adapted by W. T. W. Potts (personal communication)*

Temp. K	Temp. °C	V P ice Pa	V P water Pa	Time to evaporate 0·1 μm ice	Time to evaporate 0·1 μm water
273·1	0	610	610	$1{\cdot}45\times10^{-4}$ s	$1{\cdot}45\times10^{-4}$ s
248·1	−25	39·2	42·1	$2{\cdot}2\times10^{-3}$ s	$2{\cdot}02\times10^{-3}$ s
223·1	−50	3·93	5·0	$2{\cdot}0\times10^{-2}$ s	$1{\cdot}61\times10^{-2}$ s
198·1	−75	$1{\cdot}21\times10^{-1}$	$2{\cdot}54\times10^{-1}$	$6{\cdot}2\times10^{-1}$ s	$2{\cdot}8\times10^{-1}$ s
173·1	−100	$1{\cdot}32\times10^{-3}$	$3{\cdot}64\times10^{-3}$	54 s	19·4 s
148·1	−125	$3{\cdot}17\times10^{-6}$	$1{\cdot}22\times10^{-5}$	5·7 h	1·5 h
133·1	−140	$2{\cdot}88\times10^{-8}$	$1{\cdot}44\times10^{-7}$	25 days	5 days
123·1	−150	$6{\cdot}68\times10^{-10}$	$4{\cdot}16\times10^{-7}$	2·84 years	166 days
103·1	−170	$4{\cdot}15\times10^{-14}$	$4{\cdot}80\times10^{-13}$	$4{\cdot}18\times10^{4}$ years	$3{\cdot}61\times10^{3}$ years
93·1	−180	$7{\cdot}06\times10^{-17}$	$1{\cdot}29\times10^{-15}$	$2{\cdot}3\times10^{7}$ years	$1{\cdot}28\times10^{6}$ years
83·1	−190	$2{\cdot}68\times10^{-20}$	$8{\cdot}94\times10^{-19}$	$8{\cdot}6\times10^{10}$ years	$1{\cdot}74\times10^{9}$ years

To test this hypothesis, thin film thermocouples less than 1 mm² in area were prepared by vacuum evaporation of copper and nickel on to a block of Araldite fixed to the specimen carrier in the position normally occupied by the specimen. Measured in this way, the temperature at the specimen surface in the cold stage was −185° (88 K). A beam of 10 nA at 20 kV, i.e. more than 10 times greater than that usually selected for scanning, was focused on to the thin film thermocouple junction for 5 minutes. No rise in temperature greater than 0·1° could be detected.

Calculations derived from International Critical Tables (Egerton, 1929) (Table 1) demonstrate that at 103·1 K (−169·9°) the time required for the evaporation of 0·1 μm of water is $3{\cdot}61\times10^{3}$ years. However, these Tables are calculated on the assumption that evaporation is occurring in a perfect vacuum. In practice, the partial pressure of vapour within the microscope at an operating pressure of 2×10^{-5} torr (13·0 mPa) will *reduce* this already small rate of loss.

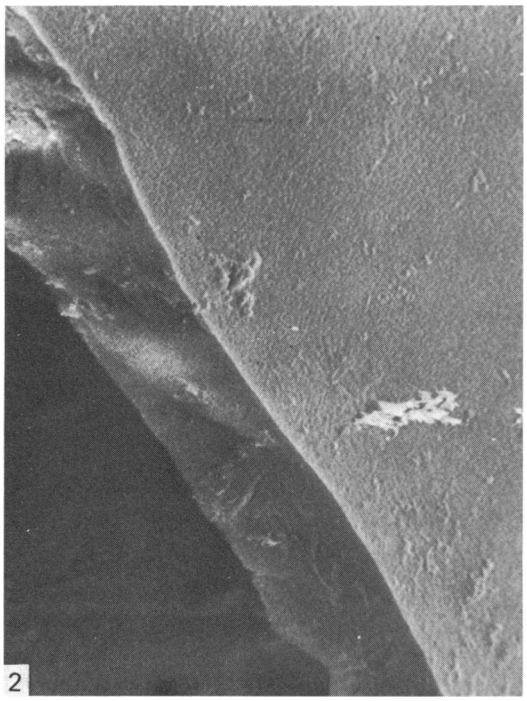
Other workers (Saubermann & Echlin, 1975) calculated the heating effect in frozen hydrated thin sections. Heating could be expected to be greater than in frozen blocks because of lower specimen thermal conductivity. An accelerating voltage of 30 kV and a beam current of 10 nA were used during 40–60 minute examinations. There was a temperature rise of 4° (4 K). No changes in residual water vapour partial pressure and total chamber pressure could be detected when measured by a quadropole mass spectrometer.

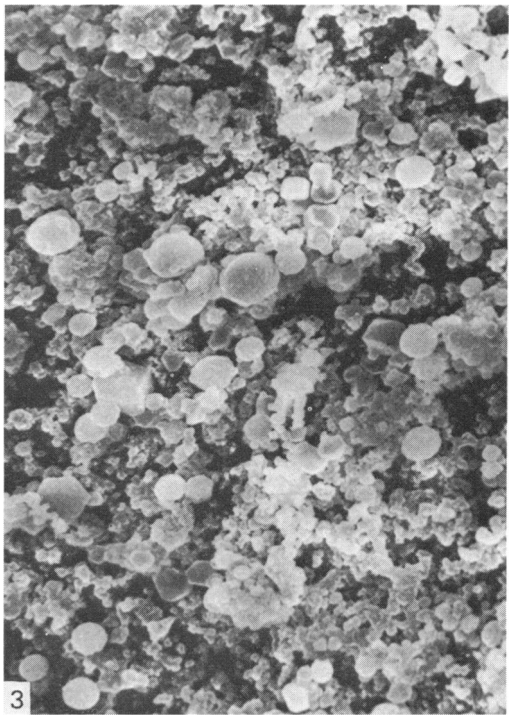
The extent of heat exchange during coating is also crucial. Thin film thermocouples have been used to measure the temperature rise on the specimen at the air lock pedestal (−181·4°) (91·6 K) where metal coating of the specimen takes place. Tests on two occasions of temperature changes during coating with aluminium, and on four occasions during coating with gold, have revealed mean rises of 12·5° (12·5 K) and of 20·0° (20·0 K) respectively. The response to radiant heat falling on a thin film thermocouple is instantaneous.

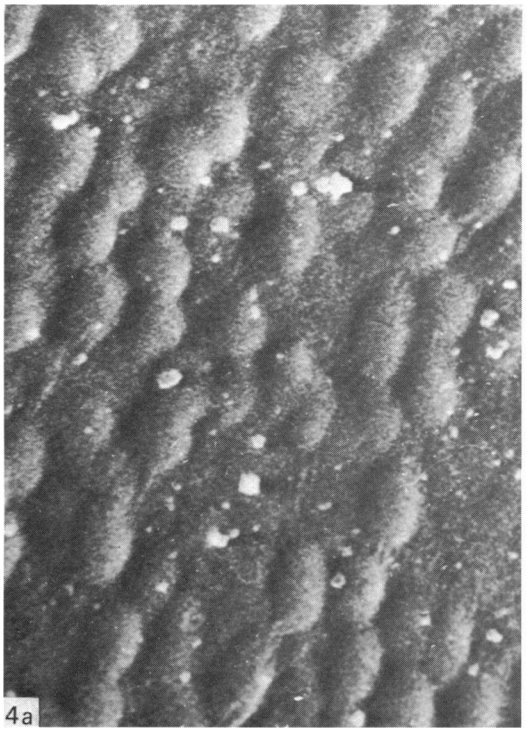
RESULTS

The edges of the blocks of dog articular cartilage were sharply defined (Fig. 2).

When specimen surfaces had been washed before block-taking, they were often but not invariably covered or obscured by deposits of a material thought to be vitrified (supercooled) fluid. The surfaces of the stubs, on which the blocks were







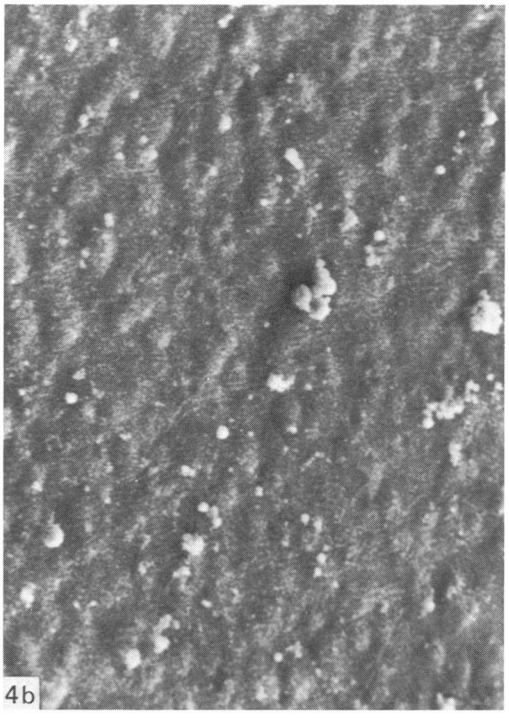


Fig. 2. Low magnification micrograph of washed dog articular cartilage showing sharply delineated cut edge of specimen. Gold coated, 60° tilt. × 70 (estimated).

Fig. 3. Frozen deposits on block of washed dog cartilage completely obliterating underlying articular surface. Gold coated. 0° tilt. × 800 (estimated).

Fig. 4. (*a*) Scattered deposits of vitrified fluid on surface of block of dog articular cartilage washed with isotonic saline. Gold coated, 45° tilt. × 340 (estimated). (*b*) Unwashed block of dog cartilage with isolated particles of frozen fluid. Gold coated, 45° tilt. × 250 (estimated).

mounted after washing, were free from this material and there was no evidence that it represented water from inside the microscope. Surfaces free from such deposits were more frequent on specimens examined without preliminary washing. However, on these surfaces, scattered discrete deposits of a different material were found; they were interpreted as residues of vitrified synovial fluid.

Washed dog cartilage

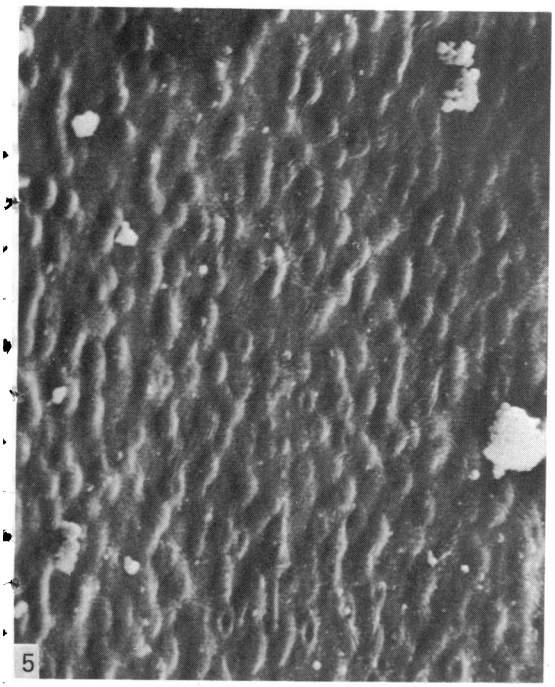
In many instances where washing had been quickly undertaken before quenching in slushy N_2, the whole or almost the whole of the free surface of the cartilage was obscured by a layer of vitrified fluid (Fig. 3). Where, by chance, a washed surface was not covered by vitrified material, the images of the surfaces closely resembled those observed in the unwashed state: aggregates of irregular particles with an estimated diameter of 10–50 μm were arranged at random on the cartilage surfaces (Fig. 4*a*). The particles appeared to be raised above the surfaces on which they were resting. The remainder of the cartilage displayed a pattern of shallow elevations recalling the undulations seen on fixed, dry cartilage surfaces (Gardner & Woodward, 1969; Inoue *et al.* 1969; Redler & Zimny, 1970; Clarke, 1971*a*, *b*; Ghadially *et al.* 1976).

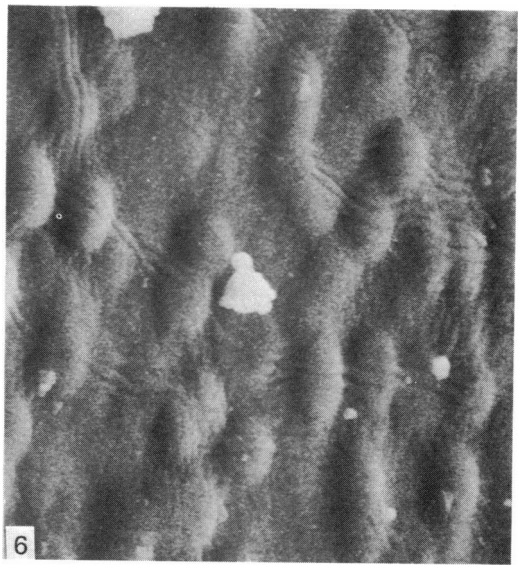
Unwashed dog cartilage

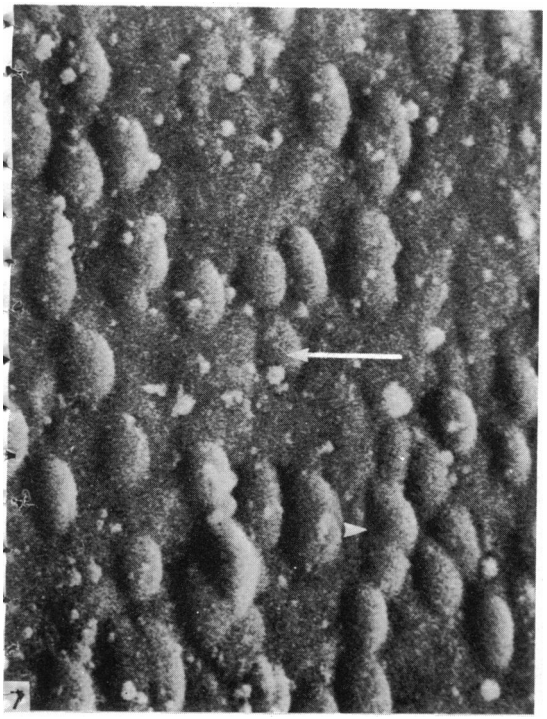
The margins of the unwashed blocks did not differ from those of washed blocks: only rarely was there recognisable compression or distortion with the formation of coarse ridges or slightly raised edges. Occasional irregular-shaped particles were present, similar to those identified on washed surfaces. These particles (Fig. 4*b*) were considered to be frozen aggregates of synovial fluid, of synovial fluid debris or of synovial fluid cells: they were similar in form to those detected on occasional washed surfaces.

The surface on which the frozen particles rested was almost uniformly covered with round, gently-contoured features that were shown by serial photographs, taken through focus, to be elevations, the 'humps' of Ghadially, Moshurchak & Thomas (1977) (Figs. 5, 6). The diameter of these elevations, thought to be $\sim$10–35 μm, could not be measured precisely since the tilt of the specimen stub (45°) necessary to secure optimum collection of secondary electrons introduced a change in specimen distance that could not be determined directly. With detailed observation, it was possible to recognise (Fig. 7) that the elevations were grouped so that areas with more prominent elevations alternated with areas in which the elevations were less conspicuous. When surfaces were scanned at a more acute angle, 3rd elevations appeared grouped in orderly rows (Fig. 8).

At higher magnification, arrays of linear ridges were detected, most conspicuous in the area between the elevations, but recognisable on the margins or even the central parts of the elevations themselves. These ridges were found in one of several arrangements: (1) short ridges, 1–2 times the diameter of the elevations, radiating from an elevation (Fig. 9); (2) long ridges, greater than 5 times the diameter of the elevations, arranged in parallel arrays across the surface of the block (Fig. 8); (3) ridges of intermediate length, 1–4 times the diameter of the elevations, forming a criss-cross pattern on the surface (Fig. 10). The mean diameter of the linear ridges was $\sim$1–2 μm; their distribution was not uniform. When the tilt angle was increased to 60°, the appearance of the arrays of linear ridges was enhanced (Fig. 8); when the angle was reduced to 30° (Fig. 11) both the 3rd elevations and the fine ridges became less conspicuous than in preparations examined with 45° or 60° tilt angles.







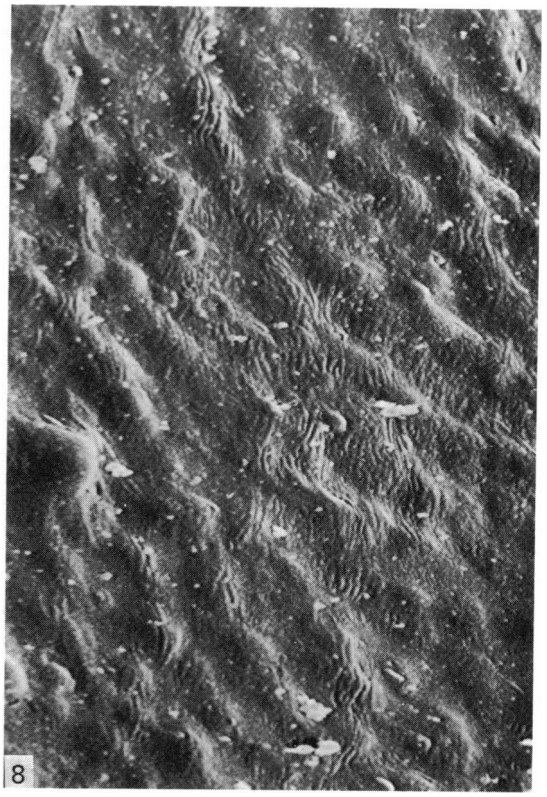
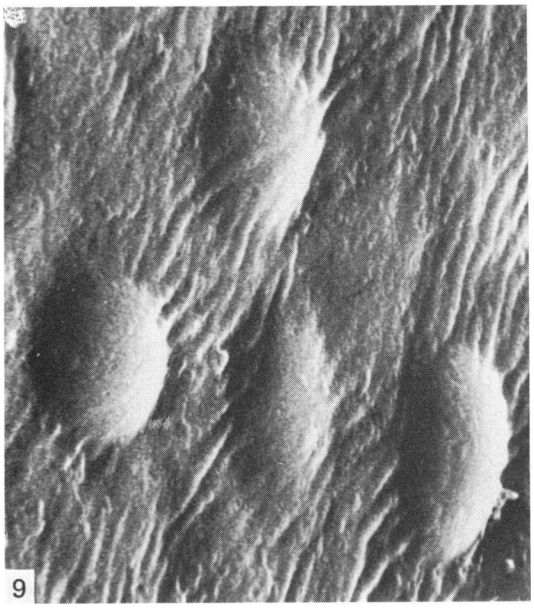


Fig. 5. Tertiary elevations cover articulating surface of block of dog cartilage unobscured by frozen material. Gold coated, 45° tilt. × 280 (estimated).

Fig. 6. Rounded tertiary prominences on unwashed specimen of dog cartilage relatively free from frozen deposits. Gold coated, 45° tilt. × 700 (estimated).

Fig. 7. In some areas of articulating surface of dog cartilage elevations are very distinct (arrowhead); in others they are much less prominent (arrow). Gold coated, 45° tilt. × 700 (estimated).

Fig. 8. At high tilt angle tertiary undulations on dog cartilage appear to form rows. Superimposed on these elevations is system of long, approximately parallel, ridges. Gold coated, 60° tilt. × 250 (estimated).



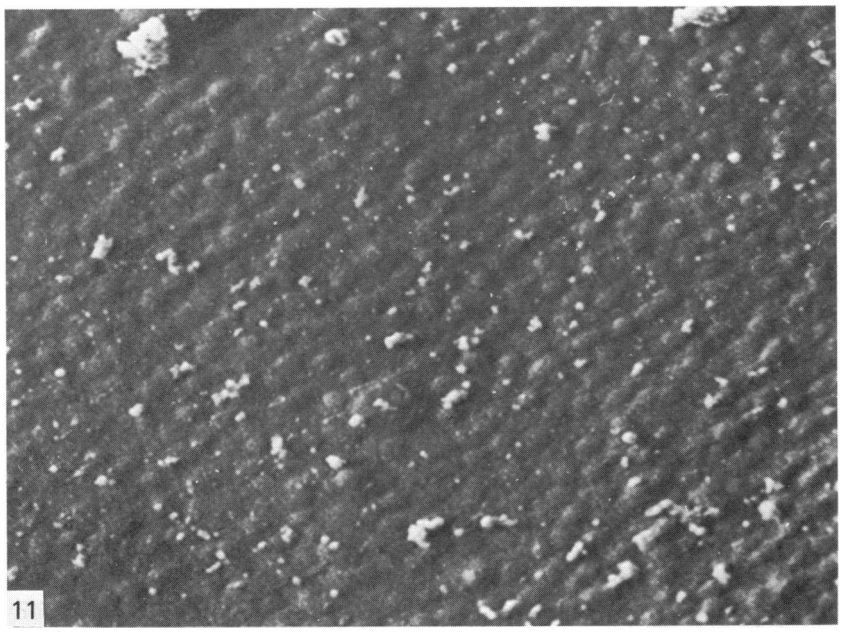
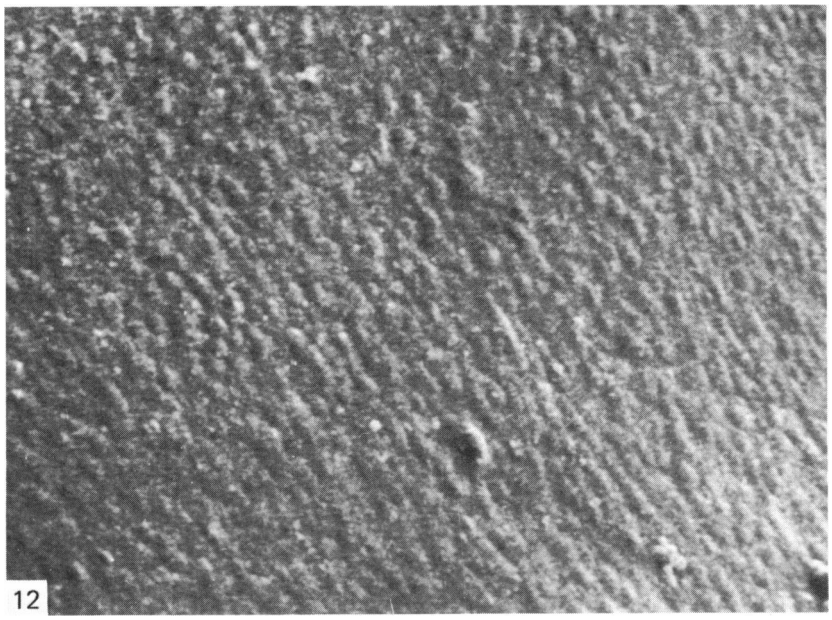
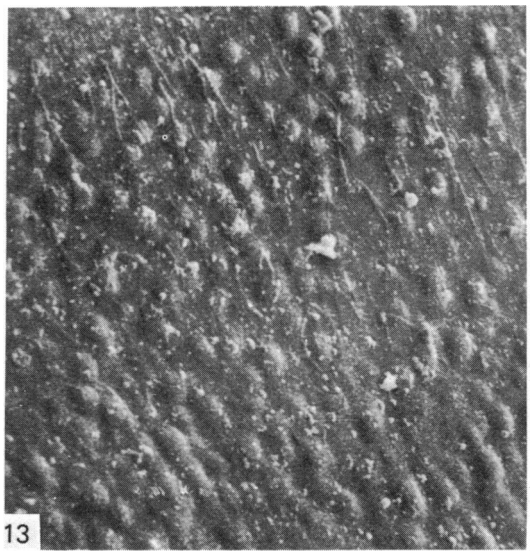


Fig. 9. Short ridges, radiating from elevations, are seen in some areas of dog articular cartilage blocks. Gold coated, 45° tilt. × 1800 (estimated).

Fig. 10. Criss-cross pattern of quaternary ridges in regions of dog cartilage between elevations and superimposed on elevations. Gold coated, 45° tilt. × 600 (estimated).

Fig. 11. Tertiary prominences on dog cartilage become less conspicuous when tilt angle is reduced. Gold coated, 30° tilt. × 150 (estimated).





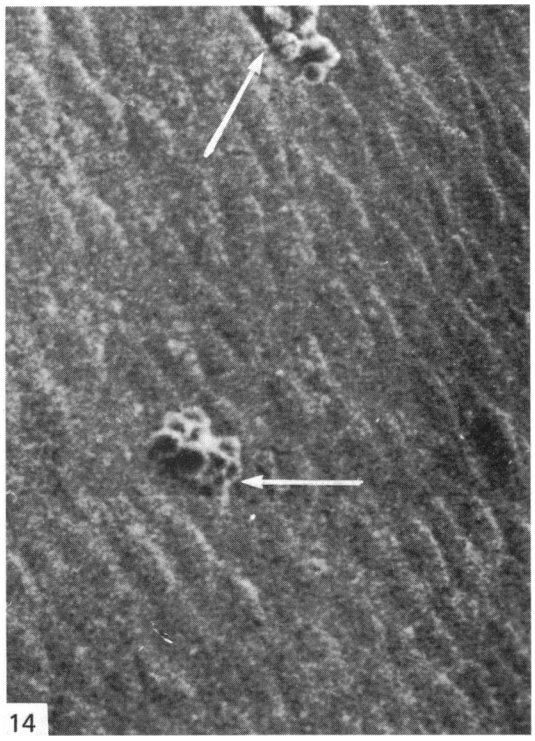


Fig. 12. Articulating surfaces of guinea-pig femoral condyles covered by tertiary undulations. Region immediately above condylar notch is illustrated; note small deposits of vitrified material. Aluminium coated. × 140 (estimated).

Fig. 13. Long, linear strands, running between and over tertiary undulations, are observed on guinea-pig articular surfaces. Aluminium coated. × 210 (estimated).

Fig. 14. Uncoated, whole guinea-pig femoral condylar cartilage surfaces have gently contoured tertiary prominences. Isolated frozen deposits (arrows) overlie these structures. Uncoated. × 380 (estimated).

Unwashed coated guinea-pig cartilage on bone

The articular surfaces of whole guinea-pig femoral condyles, on which the cartilage remained attached to the underlying bone, were very similar in appearance to the surfaces of cartilage blocks taken from dog femoral condyles. They were covered by tertiary prominences (Fig. 12). Deposits of vitrified fluid, of various sizes, were scattered over the cartilage surfaces. In some areas of the surface of the guinea-pig condyle, long isolated strands (striations), distinct from the 4^{ry} ridges of the dog material, were observed (Fig. 13).

Unwashed, uncoated guinea-pig cartilage on bone

The possibility that the structure and appearances of coated guinea-pig cartilage might be modified by the temperature changes that occur during coating was examined by the scanning, at low magnification, of cartilage surfaces that had not been subjected to this treatment. Although satisfactory images were not easily obtained, because of charging, sufficient information was derived to show that coating caused no significant alteration in surface structure. Tertiary undulations were easily detected on this tissue together with isolated deposits of super-cooled fluid (Fig. 14).

DISCUSSION

The results of the experiments described in this paper demonstrate that fully hydrated, untouched, unfixed mammalian articular cartilage can be examined effectively by low temperature scanning electron microscopy. Photographic images susceptible to constructive interpretation can be obtained at low magnification whether the bulk material has been coated or not. Reproducible results have been derived from free blocks of dog femoral cartilage, resected from the bone, and from guinea-pig femoral cartilage surfaces scanned on the whole femoral condyle. When cartilage blocks are coated before they are scanned at low temperature by the electron beam, the evidence shows that less surface damage is incurred than during conventional SEM. Finally, the evidence is strong that the 2^{ry} and 3^{ry} surface features of mammalian articular cartilage are identifiable on fully hydrated unfixed surfaces and that they are not simply artefactual products of fixation and/or dehydration as Ghadially *et al.* (1976) have repeatedly suggested. Nor are they caused by resecting cartilage from bone before freezing. The *form* of the 3^{ry} features is apparently influenced by retaining water so that a hollow or crater evinced in the dry state appears as a prominence or circumscribed elevation in the hydrated state.

Implicit in the argument that fully hydrated, non-fixed biological material can be examined constructively at very low temperature is the evidence that the surface of bulk material can be so rapidly frozen that ice crystal formation does not occur; that the material can be transported to the low temperature SEM without exposure to air (and water vapour); that coating can be effected at very low (−182°; 91 K) temperature without a rise to that (−130°; 143 K) at which the sublimation of water becomes significant; and that valid images can be obtained from the surfaces of bulk frozen material. The results of preliminary studies of articular cartilage by such low temperature methods have been published by Gardner, O'Connor & Oates (1979) and by Gardner, Oates & O'Connor (1979, 1980).

The question of whether or not the 0·5 mm 2^{ry} surface features of disarticulated hyaline cartilage and the ~30 μm 3^{ry} undulations detected on these surfaces, are

preparative artefacts, has exercised observers from the time (1968) when the roughness of articular surfaces was first suspected. The principal difficulty has been the common need to fix and to dehydrate cartilage blocks before microscopy. In recent years there has been very rapid progress in the understanding of the two and three dimensional structure of mammalian hyaline articular cartilage (Stockwell, 1979) and substantial advances in knowledge of the physical (Kempson, 1979; Maroudas, 1979) and chemical (Muir, 1978) properties of this tissue and of its behaviour *in vitro* (Fell, 1978). However, much of the information regarding the morphology of cartilage viewed in section by light microscopy (LM), by transmission electron microscopy (TEM) or by high voltage TEM (Glauert, 1979) has been derived from fixed, dehydrated preparations; and this criticism is applicable to almost the whole of the data concerning the three dimensional structure of hyaline articular (Ghadially *et al.* 1976) and of elastic (Holm Nielsen & Bytzer, 1979) cartilage surveyed by SEM. To a varying extent, the disadvantages of working with fixed, dehydrated material have been overcome by selecting cryostat sections for phase contrast LM or fresh, unfixed blocks of tissue for RLIM (Longmore & Gardner, 1975; 1978). However, the significance of the results obtained by these methods has been limited by the wavelength of visible light; those derived from phase contrast LM have been criticised for other reasons. Thus, the *lamina splendens* (MacConaill, 1951) has been shown to be artefactual (Aspden & Hukins, 1979). In the course of electron probe X-ray microanalysis of tissues other than cartilage, unfixed, thin sections have been studied (Saubermann & Echlin, 1975) at high resolution but, until recently, no satisfactory method has been available for the investigation by SEM of non-fixed, fully hydrated hyaline cartilage surfaces. This is of particular importance because of the critical part that water plays in determining the physical properties of cartilage, and because of growing evidence that fixation in formaldehyde (Wilson & Gardner, 1980), or in glutaraldehyde, and freeze drying (Sayles *et al.* 1979) cause significant, measurable shrinkage and distortion of cartilage blocks.

These technical limitations have proved a serious problem in the interpretation of microscopic images of disarticulated, non-loaded hyaline cartilage surfaces. Nevertheless, by 1971, a classification of the morphological surface features of mammalian hyaline articular cartilage had been proposed (Gardner & MacGillivray, 1971) that described (1) 1^{ry} anatomical contours; (2) 2^{ry} ($\sim$0·5 mm diameter) irregularities; and (3) 3^{ry} ($\sim$10–40 μm diameter) undulations. These features were recognisable by naked eye; by hand lens; and by RLM and SEM, respectively. Subsequently, fine arrays of delicate 4^{ry} irregularities on free, non-marginal human cartilage surfaces were identified by the technique of RLIM (Longmore & Gardner, 1975; 1978).

The presence of the 1^{ry} and 2^{ry} features cannot seriously be questioned: they are seen on living and non-living cartilage surfaces, whether or not the cartilage has been detached from bone and irrespective of the state of hydration of the specimen. Tertiary undulations were first identified on the non-loaded surfaces of the femoral condyles of whole guinea-pig joints, by LM (Gardner & Woodward, 1969). The presence of these 3^{ry} features has been accepted by the majority of reviewers (McCutchen, 1978; Meachim & Stockwell, 1979; Stockwell, 1979; Ghadially *et al.* 1976): they can be seen on the untouched surfaces of infant and adult human, and on baboon, dog, rabbit, guinea-pig, rat, mouse, fetal mouse and turkey cartilage. The evidence showing that 3^{ry} undulations exist on the non-loaded surfaces of the cartilage of diarthrodial joints is consequently very strong.

If the knee joint of the guinea-pig is carefully disarticulated immediately after

killing, and the free cartilage surface of the femoral condyles examined with an operating, dissecting or incident light microscope, the shallow 3^{rv} undulations can be readily shown by low angled incident light, confirming observations made in 1968 (Gardner & Woodward, 1969). The presence of the undulations on fresh unfixed surfaces from whole joints can be substantiated by making 1-stage latex replicas. The undulations can be measured as Talysurf traces of the replicas; or traces can be made directly from the cartilage (Sayles *et al.* 1979). Alternatively, the cartilage undulations can be thrown into relief by immersing a whole fresh bone in buffered osmium tetroxide. The undulations can be detected on living articular surfaces (Gardner & McGillivray, 1971) and have been observed and measured *in vitro* on excised cartilage by RLIM (Longmore & Gardner, 1975; 1978), a method that has allowed the demonstration that the undulations increase in depth and diameter with age, but that, with time, their frequency diminishes. Although the undulations may, in theory, result from some rapid change, such as water loss, occurring between the time of joint exposure and microscopy (Meachim & Stockwell, 1979), this seems unlikely since undulations can be seen on the surface of young adult human femoral condyles viewed in the operating theatre during surgery (Gardner & McGillivray, 1971). However, it is necessary to remember that exploratory arthrotomy of the human knee is commonly undertaken in a bloodless field secured by the application of a femoral tourniquet. Deprivation of blood flow to a limb in the living anaesthetised rat has a remarkable effect on the appearance of exposed femoral condylar cartilage surfaces (Gardner & McGillivray, 1971). Furthermore, small blocks of excised cartilage lose water very quickly indeed (Kirkpatrick, 1977) and the possible effects of the rapid drying of exposed cartilage surfaces must always be considered.

It is consequently of very great interest that shallow 3^{rv} features of cartilage are readily detected on the surface of non-loaded guinea-pig femoral condyles that have been taken into slushy N_2 at $-210°$ (63 K) and examined by SEM at $-194°$ (79 K) without detachment from the underlying bone, without fixation and without dehydration. The evidence does not explain the reason for the existence of 3^{rv} features on disarticulated surfaces or the relationship of the undulations to joint lubrication. Nor can it be stated categorically that the undulations, which probably exist *in vivo*, are invariably present on loaded, opposed living articular surfaces although limited direct experimental evidence that this may be the case has been presented (Gardner, 1972). Clarke (1971*b*) suggested that cartilage surface micro-contours may be continually changing over short periods of time. It is possible that such changes, related in the living state either to surface lubrication or to cartilage nutrition (Stockwell, 1979), may permit 3^{rv} undulations to be more or less prominent *in vivo* according to the functional state of the tissue. We are not aware of published evidence to confirm or refute this view.

There is a growing recognition of the influence of fixation and of drying on the appearances of bulk material and of sections examined by SEM and TEM respectively. The use of low temperature techniques has emerged less because of the need to avoid fixation and drying artefact than because of a wish to retain water, electrolytes and other small molecules in a normal, *in vivo* spatial relationship. However, success in freezing and in low temperature microscopy now underlines the effects of drying and fixation on fine structure. In the present experiments, it is apparent that the 2^{rv} and 3^{rv} surface features detectable by LM, RLIM, by SEM and by replication, can also be identified by low temperature SEM. However, the 3^{rv} undulations recognised by low temperature SEM are not of the same size and shape as those found on

the surfaces of fixed, dry SEM preparations. On the surface of dog specimens, the 3^{ry} features are predominantly elevations; the degree of elevation varies. Measurement of the exact size of surface features has been restricted because of difficulty in determining specimen height at the tilt angles necessary to image 3^{ry} undulations, and must await advances in instrumentation.

During early experiments, it was suspected that washing cartilage surfaces at the time joints were opened led to an increase in the deposition of vitrified fluid. There was no ice on the corresponding metal stub: fluid was assumed, therefore, to have frozen on the cartilage at the moment of initial immersion in slushy nitrogen and not at a later stage in specimen preparation or examination. To avoid this latter form of subsequent surface contamination has been the main purpose of the sophisticated transfer systems that have been designed to allow material to be moved from the slushy nitrogen to the electron microscope or its antechamber without exposure to atmospheric moisture. This surface deposit and that exaggerated by washing could, of course, be removed by surface sublimation (etching) without risk of significant change in the structure of the underlying cartilage. A variable amount of vitrified fluid is also recognised on rapidly frozen unwashed cartilage surfaces: it has been deduced that this may be synovial fluid. Etching has therefore also been used in a small number of selected instances to remove this layer. The underlying surfaces have been found to display morphological features at low magnifications that closely resemble those seen on surfaces examined without this treatment. However, a particular problem in the examination of the free cartilage surfaces of diarthrodial joints, and one for which no absolute solution can be anticipated, is that these surfaces are, by definition, moist. The loss or attempted removal of surface synovial fluid could be said to impair the integrity of the surface bone–cartilage–synovial fluid–cartilage–bone continuum (Gardner, Elliott, Gilmore & Longmore, 1975) and thus to alter the normality of the very structure which it is intended to examine. It is likely that the loss of water by evaporation during specimen collection can be minimised by the use of a humidity chamber.

The reasonable suggestion has been made that resecting blocks of cartilage from underlying bone during specimen collection introduces material distortion and artefact. This proposal seeks in part or whole to explain (1) the gross marginal compression of cartilage blocks (Clarke, 1971*a*); (2) the appearances of 3^{ry} undulations; and (3) the recognition of quarternary (4^{ry}) ridges (Ghadially *et al.* 1976), on the basis of this mechanical disturbance.

There is no doubt that the preparation of cartilage blocks, even with the sharpest blade, compresses the margins of the material and introduces limited distortion (Ghadially *et al.* 1976). However, the coarse 60–80 μm ridges that are easily produced can, in large part, be avoided by careful technique: they do not extend across the frozen material and are readily distinguished from fine 3^{ry} undulations and 4^{ry} ridges. Marginal distortion is prevented by collecting whole small joints with the cartilage remaining on the bone, a technique not applicable to the examination in the present generation of low temperature SEM of larger whole joints. Tertiary undulations are reproducibly identified and investigated on free cartilage surfaces, whether or not the material is derived from resected or intact joints. The resection of cartilage from bone is not responsible for the production of these 3^{ry} surface features. The relationship of compression and distortion to the presence and distribution of the finer 4^{ry} ridges described both by RLIM and by SEM is less easy to interpret. Quarternary ridges, in our experience, are recognised only on the surfaces of material

that has been detached from bone during collection: the ridges were seen on dog cartilage blocks in the present study but not on guinea-pig. The blocks of human cartilage surveyed by Longmore & Gardner (1975; 1978) had also been freed from underlying bone.

The possibility that the methods described in this paper for the investigation of cartilage by low temperature SEM introduce previously unrecognised artefacts must also be considered. Rates of tissue freezing by quenching have been measured (Bald, 1975; Bald & Robards, 1978); there is a linear relationship between rate of heat loss and the surface area: volume ratio of the bulk material. Since the present experiments are concerned with surface structure, and since heat loss from the superficial 1–2 μm of cartilage is so rapid at a controlled environmental temperature of $-210°$ (63 K) (the temperature of slushy nitrogen) as to be measurable by only the most sensitive instruments, there appears no reason to believe that surface morphology can be artefactually changed. However, the *differential* loss of heat from the various cartilage layers is less easy to measure. When larger, non-cryoprotected blocks are quenched in a very low temperature fluid, $\not> 12$ μm of surface material is believed to lose heat so rapidly that freezing can be shown to be unaccompanied by electron microscopically visible ice crystals (Dempsey & Bullivant, 1976*a*, *b*).

The possibility must therefore be acknowledged that differential cooling between, say, the most superficial 12 μm and the subjacent 10–12 μm of cartilage may cause mechanical stresses that could lead to distortion. However, the gradient of heat exchange is low. It is also suspected that chondrocytes, the pericellular matrix and the intercellular matrix, each cool at different rates and that microstresses develop in the cellular environment that could cause changes in fine structure. At the resolution attainable in the SEM, the structural changes resulting from these gradients are not likely to be detectable. In preliminary experiments with blocks that have been retrieved for light microscopy after low temperature SEM, no alterations in the mid- and deeper zone cartilage have been recognised.

The possibility that large surface artefact detectable by SEM is caused during the coating of bulk material at very low temperature can now be discounted. Direct measurements made at the surfaces of model blocks of gelatine and of blocks of hyaline cartilage show that material held at $-182°$ (91 K) in the front chamber of the JSM 50A microscope is subjected to a rise of $\sim 12°$ during coating with aluminium, of $\sim 20°$ with gold, and of $\sim 25°$ during carbon coating. Since the sublimation of water becomes significant only at temperatures $> -130°$ (143 K), and since thermal damage of cartilage surfaces by the coating process has not been recognised, there is no reason to think that coating contributes to the presence or absence of any of the large surface features described in this paper. This view is supported by present evidence in which coated and uncoated material have been directly compared.

Closely similar arguments determine the answer to the question: does prolonged examination by the electron beam during SEM, alter or distort surfaces held at very low ($\sim -195°$; 78 K) temperatures? Direct measurements made by thin film thermocouple reveal that the loss of heat from a cartilage block held at $-195°$ (78 K) is so rapid that no temperature change is measurable during prolonged scanning with beam currents ten times greater than those customarily selected. The system can be said to constitute a 'heat sink'.

From material treated in the manner described in this paper, it is concluded that surface features can be imaged to permit comparison with those described by conventional SEM techniques, by RLIM, by replication and by Talysurf tracing.

The way now appears open for the investigation of similar material at increasing magnification with the aim of resolving surface detail both in normal and in abnormal tissue. Many other aspects of histology and of histopathology that are contentious may, it is anticipated, be re-examined by analogous low temperature methods. Arterial endothelium, chorionic villi, intestinal epithelium, the cornea, the choroid and the skin all lend themselves to this suggestion. There is little doubt that preparative techniques will be modified and improved as these proposals are explored and as new instruments become available.

SUMMARY

Fifty seven blocks of cartilage excised from the femoral condyles of 20 beagle dogs, and whole lower ends of 5 guinea-pig femora, were examined at $-195°$ (78 K), by scanning electron microscopy. The unfixed tissue, taken into slushy nitrogen at $-210°$ (63 K), was not exposed to atmospheric air after quenching and remained fully hydrated throughout long periods of observation.

Images susceptible to analysis were obtained from washed and from unwashed cartilage surfaces. Preliminary coating with gold or with aluminium, known to be possible without exposing cold cartilage surfaces to changes in temperature likely to cause water loss by sublimation, was valuable in minimising charging and in facilitating the recording of electron images at higher magnifications. Although examination was possible without coating, the resultant images were of low resolution.

Microscopy revealed a pattern of secondary surface irregularities and of tertiary elevations closely resembling those seen by the conventional scanning electron microscopy of fixed, dehydrated hyaline cartilage. However, the pattern of tertiary surface structures was predominantly that of elevations, not of hollows. Quarternary surface ridges were common on the surfaces of excised dog cartilage blocks and were not seen on the surfaces of guinea-pig cartilage which remained on the femoral condyles.

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REFERENCES

ASPDEN, R. M. & HUKINS, D. W. L. (1979). The lamina splendens of articular cartilage is an artefact of phase contrast microscopy. *Proceedings of the Royal Society, B* **206**, 109–113.

BALD, W. B. (1975). A proposed method for specifying the temperature history of cells during the rapid cool-down of plant specimens. *Journal of Experimental Botany* **16**, 103–119.

BALD, W. B. & ROBARDS, A. W. (1978). A device for the rapid freezing of biological specimens under precisely controlled and reproducable conditions. *Journal of Microscopy* **112**, 3–15.

CLARKE, I. C. (1971*a*). Surface characteristics of human articular cartilage – a scanning electron microscope study. *Journal of Anatomy* **108**, 23–30.

CLARKE, I. C. (1971*b*). Human articular surface contours and related surface depression frequency studies. *Annals of the Rheumatic Diseases* **30**, 15–23.

DEMPSEY, G. P. & BULLIVANT, S. (1976*a*). A copper block method for freezing non-cryoprotected tissue to produce ice crystal-free regions for electron microscopy. I. Evaluation using freeze substitution. *Journal of Microscopy* **106**, 251–260.

DEMPSEY, G. P. & BULLIVANT, S. (1976*b*). A copper block method for freezing non-cryo-protected tissue to produce ice crystal-free regions for electron microscopy. II. Evaluation using freeze fracturing with a cryo-ultramicrotome. *Journal of Microscopy* **106**, 261–277.

ECHLIN, P., RALPH, B. & WEIBEL, E. R. (1978). *Low Temperature Biological Microscopy and Microanalysis*. Oxford, London and Edinburgh: Blackwell Scientific Publications.

EGERTON, A. C. (1929). *International Critical Tables* 5.53. New York, London: McGraw-Hill.

FELL, HONOR B. (1978). Synoviocytes. In *Diseases of Connective Tissue* (ed. D. L. Gardner). *Journal of Clinical Pathology* **31**, Suppl. (Royal College of Pathologists) **12**, 14–24.

GARDNER, D. L. (1972). The influence of microscopic technology on knowledge of cartilage surface structure. *Annals of the Rheumatic Diseases* **31**, 235–258.

GARDNER, D. L., ELLIOTT, R. J., GILMORE, R. ST. C. & LONGMORE, R. B. (1975). Microscopical appearances and organization of articular cartilage surfaces. *Annals of the Rheumatic Diseases* **34**, Suppl. pp. 2–4.

GARDNER, D. L. & MCGILLIVRAY, D. C. (1971). Living articular cartilage is not smooth. *Annals of the Rheumatic Diseases* **30**, 3–9.

GARDNER, D. L., OATES, K. & O'CONNOR, P. (1979). Surface structure of mammalian articular cartilage: an investigation by low temperature scanning electron microscopy. *Clinical Science* **58**, 31P.

GARDNER, D. L., OATES, K. & O'CONNOR, P. (1980). Low temperature scanning electron microscopy (SEM) of tissue surfaces. *Proceedings of the 140th Meeting of the Pathological Society of Great Britain and Ireland*, p. 46 (paper 61).

GARDNER, D. L., O'CONNOR, P. & OATES, K. (1979). Surface structure of mammalian articular cartilage viewed at low temperature. *Lancet* **II**, 538.

GARDNER, D. L. & WOODWARD, D. (1969). Scanning electron microscopy and replica studies of articular surfaces of guinea-pig synovial joints. *Annals of the Rheumatic Diseases* **28**, 379–391.

GHADIALLY, F. N., GHADIALLY, J. A., ORYSCHAK, A. F. & YONG, N. K. (1976). Experimental production of ridges on rabbit articular cartilage: a scanning electron microscope study. *Journal of Anatomy* **121**, 119–132.

GHADIALLY, F. N., MOSHURCHAK, E. M. & THOMAS, I. (1977). Humps on young human and rabbit articular cartilage. *Journal of Anatomy* **124**, 425–435.

GLAUERT, A. M. (1979). Recent advances of high voltage electron microscopy in biology. *Journal of Microscopy* **117**, 93–102.

HOLM NIELSEN, E. & BYTZER, P. (1979). High resolution scanning electron microscopy of elastic cartilage. *Journal of Anatomy* **129**, 823–831.

INOUE, H., KODAMA, T. & FUJITA, T. (1969). Scanning electron microscopy of normal and rheumatoid articular cartilages. *Archivum histologicum japonicum* **30**, 425–435.

KEMPSON, G. E. (1979). Mechanical properties of articular cartilage. In *Adult Articular Cartilage* (ed. M. A. R. Freeman), 2nd edition. pp. 333–414. London: Pitman Medical.

KIRKPATRICK, C. J. (1977). Organ culture techniques in the study of cartilage growth. Ph.D. thesis. The Queen's University of Belfast.

LONGFIELD, M. D., DOWSON, D., WALKER, P. S. & WRIGHT, V. (1969). 'Boosted lubrication' of human joints by fluid enrichment and entrapment. *Bio-medical Engineering, London* **4**, 517–522.

LONGMORE, R. B. & GARDNER, D. L. (1975). Development with age of human articular cartilage surface structure. *Annals of the Rheumatic Diseases* **34**, 26–37.

LONGMORE, R. B. & GARDNER, D. L. (1978). The surface structure of ageing human articular cartilage: a study by reflected light interference microscopy (RLIM). *Journal of Anatomy* **126**, 353–365.

MCCALL, J. C. (1968). Scanning electron microscopy of articular surfaces. *Lancet II*, 1194.

MACCONAILL, M. A. (1951). The movement of bones and joints. 4. The mechanical structure of articulating cartilage. *Journal of Bone and Joint Surgery* **33B**, 251–257.

MCCUTCHEN, C. W. (1978). Lubrication of Joints. In *The Joints and Synovial Fluid*, Vol. I. (ed. L. Sokoloff) pp. 438–483. New York: Academic Press.

MAROUDAS, A. (1979). Physicochemical properties of articular cartilage. In *Adult Articular Cartilage* (ed. M. A. R. Freeman), 2nd edition. pp. 215–290. London: Pitman Medical.

MEACHIM, G. & STOCKWELL, R. A. (1979). The matrix. In *Adult Articular Cartilage* (ed. M. A. R. Freeman), 2nd edition. pp. 16–21. London: Pitman Medical.

MUIR, I. H. M. (1978). Proteoglycans of cartilage. In *Diseases of Connective Tissue* (ed. D. L. Gardner), *Journal of Clinical Pathology* **31**, Suppl. (Royal College of Pathologists) **12** pp. 67–81.

REDLER, I. & ZIMNY, M. J. (1970). Scanning electron microscopy of normal and abnormal articular cartilage and synovium. *Journal of Bone and Joint Surgery* **52A**, 1395–1404.

SAUBERMANN, A. J. & ECHLIN, P. (1975). The preparation, examination and analysis of frozen hydrated tissue sections by scanning transmission electron microscopy and X-ray microanalysis. *Journal of Microscopy* **105**, 155–191.

SAYLES, R. S., THOMAS, T. R., ANDERSON, J., HASLOCK, I. & UNSWORTH, A. (1979). Measurement of the surface microgeometry of articular cartilage. *Journal of Biomechanics* **12**, 257–267.

STOCKWELL, R. A. (1979). *Biology of Cartilage Cells*, pp. 158–161. Cambridge University Press.

TAYLOR, P. G. & BURGESS, A. (1977). Cold stage for electron probe microanalyser. *Journal of Microscopy* **111**, 51–64.

WILSON, N. H. F. & GARDNER, D. L. (1980). Influence of aqueous fixation on articular surface morphology. A reflected light interference microscope study. *Journal of Pathology* **131**, 333–338.