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EARLY SCANNING ELECTRON MICROSCOPIC STUDIES OF HARD TISSUE RESORPTION: THEIR RELATION TO CURRENT CONCEPTS REVIEWED

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Abstract

This paper highlights some observations made by the authors in SEM studies of hard tissue resorption and considers their significance in relation to current concepts. All mammalian mineralised tissues may undergo physiological resorption, the resulting surface reflecting the density of mineralisation and the organic matrix chemistry, organisation and orientation. Resorption-repair coupling may follow the resorption of any tissue, but SEM studies first noted this process in the case of the dental tissues. The difference between fetal and adult boneformation and resorption provided evidence against the concept of osteocytic osteolysis. SEM stereophotogrammetric methods for the quantitation of individual resorption lacunae are now much quicker and have been extended to the study of in vitro resorption by mammalian and avian osteoclasts isolated from bone and seeded into new substrates.

Experimental studies using SEM were first conducted on the osteotropic hormonal effects on bones forming in vivo and extended to the in vitro situation. The effects observed underlined the several actions of PTH on osteoblasts and indicated their important role in the control of bone resorption. Immunological marking techniques monitored by SEM first established that osteoclasts had no Fc or C_3 receptors, although other cells in the vicinity did.

The study of osteoclasts resorbing substrates other than bone in vitro has increased our understanding of the essential components of a resorbable substrate. Experiments growing separated bone cells and marrow cells on calcified substrates have shown that such cells will continue to resorb for at least six weeks.

KEY WORDS: Bone disease, resorption, osteoclasts, osteoblasts, scanning electron microscopy, stereophotogrammetry, enamel, dentine, cementum.

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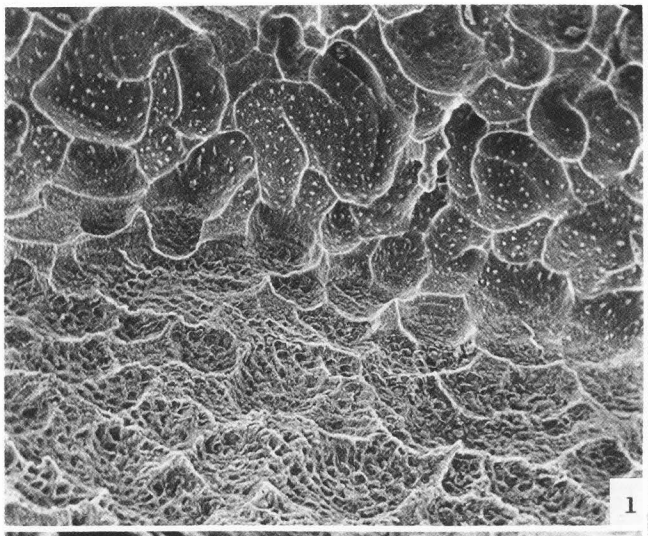
Introduction

This paper was presented as the lead-off talk at a meeting (Biomineralization: Edward J. Reith Memorial Meeting on Scientific Investigation of Vertebrate Mineralized Tissues) held at New Orleans, May 5-7, 1986. This meeting was organized with the purpose of reviewing some of the major areas in which the SEM has contributed to understanding of hard tissue structure and function in vertebrates. The present paper sets out to examine one small facet of this microscopic-method-well-suited-to-the-problem area, and from one direction only. We have taken a second look at our own earlier SEM data to see how it has affected our viewpoints concerning the natural history of resorption, and how the SEM study slots in with other recent cell biological studies.

The morphology of resorption

The phenomenon of osteoclastic resorption occurs in all the calcified tissues: not only bone, and calcified cartilage, but all the dental tissues. The first published SEM study of resorption concerned the dentine and enamel of human deciduous teeth <1>. 3-D images of resorption surfaces at a range of magnifications showed that differential rates of removal reflected local compositional differences in these tissues. For example, within the "floors" of individual resorption lacunae, peritubular dentine (PTD) stood proud of the surrounding, mineralized-collagen intertubular dentine. In enamel, prism boundary regions stood above the rest of the enamel structure: at the enamel-dentine junction (EDJ), the enamel stood slightly above dentine (Fig. 1). These studies showed that the rate of resorption of a hard tissue is influenced by local variations in composition reflecting both the organic matrix chemistry and the density of mineralization (Fig. 2). Referring to later studies in which it has been surmised that collagen breakdown products are chemotactic for osteoclast precursors <2> and in which the degradation of the collagen has played a major role in thought processes <3>, we need to emphasize that osteoclastic resorption can continue in the absence of a collagenous component to a matrix: there is none of this protein in enamel or human PTD.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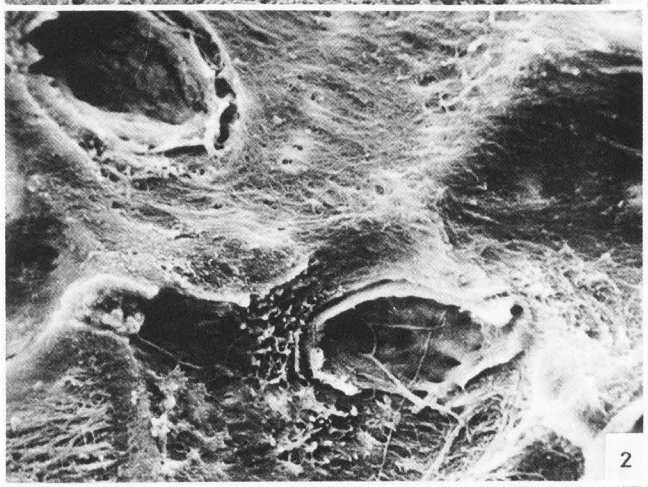
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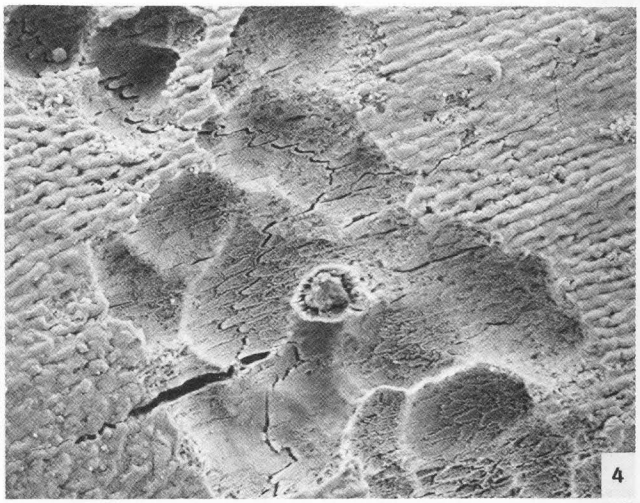
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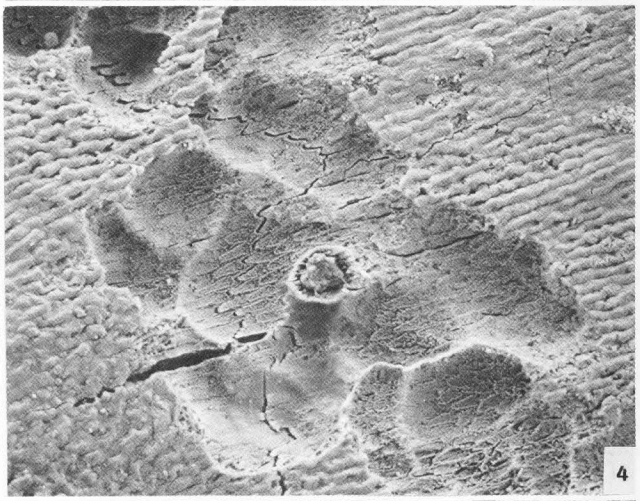
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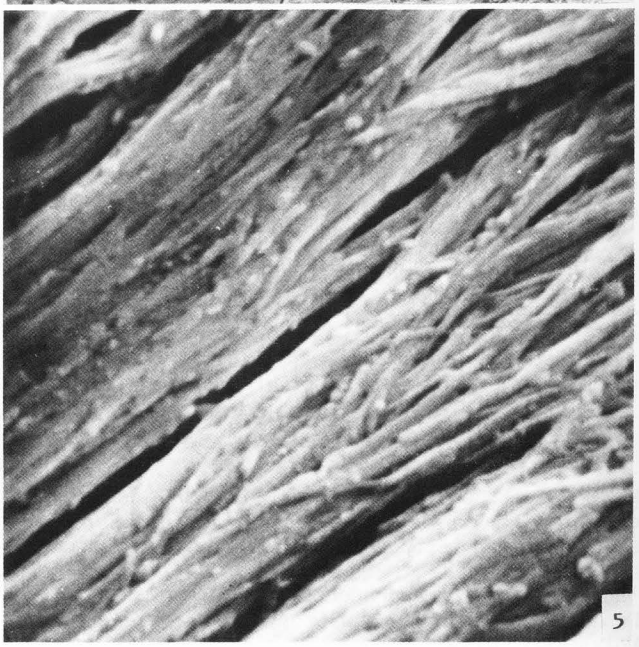
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Figure 1. Resorption of human deciduous dentine (top) and enamel prior to shedding a tooth. Fieldwidth = 296 μm.

Figure 2. Rat compact bone osteocyte lacunae exposed by osteoclastic resorption showing retention of separate perilacunar matrix phase, common in this species. Fieldwidth = 55 μm.

Figure 3. Human tooth root near apex showing patches of resorption and areas of repair emanating in prior resorbed areas. Fieldwidth = 1122 μm.

Figure 4. Horse molar enamel resorbed by osteoclasts prior to cementum deposition: another example of resorption formation coupling. Fieldwidth = 172 μm. (Stereo-pair).

Figure 5. The lining surface of a Haversian canal in adult rat mandible, made anorganic with 1,2 ethane diamine, showing complete mineralisation of matrix surface fibres in this resting surface. Fieldwidth = 7.3 μm.

Studies of human and other mammalian cementum resorption <4> showed that some resorption of the roots of human permanent teeth is a perfectly normal occurrence. Indeed, the growth of a second-phase of cementum - which may or may not contain included cells (cementocytes), but which otherwise usually includes a high proportion of extrinsic (Sharpey) fibre bundles - was shown to occur in relation to small patches of resorption in the apical portion of the root (Fig. 3). This was the first demonstration of resorption-formation coupling in an extra-skeletal site. This now popular concept was first proposed for bone on the basis of human metabolic data <5>. (Another dental example of resorption-formation coupling will be considered later - see Fig. 4).

The first SEM studies of adult and fetal bone resorption <6,7> again showed how the nature of SEM observations could bring to our attention some important facts. It was found that resorption lacunae in bone were much smaller than in deciduous tooth root removal <8>. It was noted that osteoclastic resorption left osteocyte lacunar walls intact. This observation indicated that osteocytes survived until release by resorption: they did not themselves remove either mineral or matrix as was hypothesised in the then popular concept of osteocytic osteolysis <9,10>. Another early SEM finding was that resorbed areas not uncommonly finished within fields with mineralizing fronts indicating that resorption had occurred into bone covered with osteoid <6,7,10>: the latter point was, and is still, at odds with current notions of bone pathologists who felt, and feel, that osteoid does not resorb. The most recent studies have confirmed the initial findings - osteoid can be resorbed by osteoclasts <11>. The most conservative explanation of this which we can offer today is to hypothesize that osteoclasts are able to resorb osteoid, but do not prefer to do so. Thus active, activated osteoclasts continue resorbing a surface, perhaps until they reach an osteoid covered patch; when the "stimulation" to continue resorption is reduced - but only after some osteoid has been tested or tasted. The degree of maturation of the osteoid and its thickness may also be important.

The early studies also showed that most human adult bone surfaces entered a "resting" condition, in which the collagen fibre bundles of the matrix were fully mineralized <6,7,10,12>. Thus intact fibres and bundles could be demonstrated in anorganic specimens (Fig. 5). This, and the fact that much endosteal (Fig. 6) and trabecular (Fig. 7) resorption occurs in patches within fields of well mineralized collagen, showed that osteoclasts do not have to contend with a barrier of a thin osteoid layer in order to recognize real bone <13>. Close examination of our early SEM figures of anorganic preparations <6> shows that some endosteal surfaces showed nearly, but not quite complete mineralization: some resorption patches can be found ending within such areas. However, as just pointed out, some end in osteoid proper.

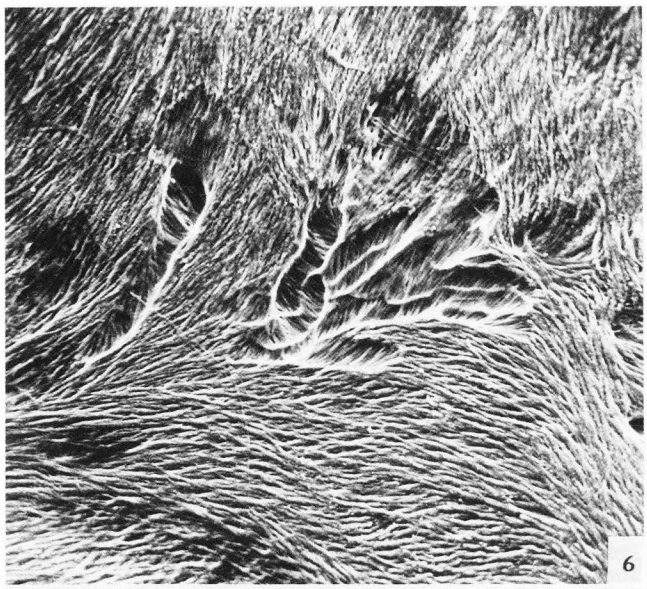
It is obvious that one of the great advantages of the SEM is the apparent 3-D nature of the 2-D projection of a surface, making it abundantly clear as to the morphological complexity of a resorbing surface. However, a 2-D projection can deceive, and we have always felt the need for a real 3-D image in our work. Stereopair images not only convey a vivid impression of relationships, but can be formally analysed to reconstruct profile sections and contour maps <14-16>. We have continued to develop stereophotogrammetric methods for the purpose of providing a quantitative description of bone resorption <17-19>.

Horse enamel

Horses have high crowned teeth which erupt and function before a root is formed. The mechanism of tooth attachment is that the crown enamel is covered with cementum, which serves to attach the fibres of the periodontal ligament. This route is adopted by many mammals, but it is usual for cement to attach to a naturally rough, pitted surface of enamel. In horses, however, the completed enamel surface, after maturation-mineralization, is superficially resorbed by osteoclasts (Fig. 4). The reduced, post-maturation ameloblasts have somehow to move or be moved out of the way for this purpose. This resorption is repaired immediately by the formation of cement. This is another example of resorption-formation coupling. However, this is most unusual in cement coupling to enamel <20>.

Hormone experiments on bone

Experiments using the SEM to observe the effects of the administration of parathyroid hormone (as parathyroid extract, PTE) and calcitonin (CT) to rats <21> showed both the power of the SEM as an observational tool, and the rapidity with which dramatic changes can be induced in bone matrix and mineral morphology. Resorption induced by parathyroid hormone (PTH) was observed to begin, at flat bone surfaces, close to the places where these were penetrated by blood vessel canals (Fig. 8). A great deal of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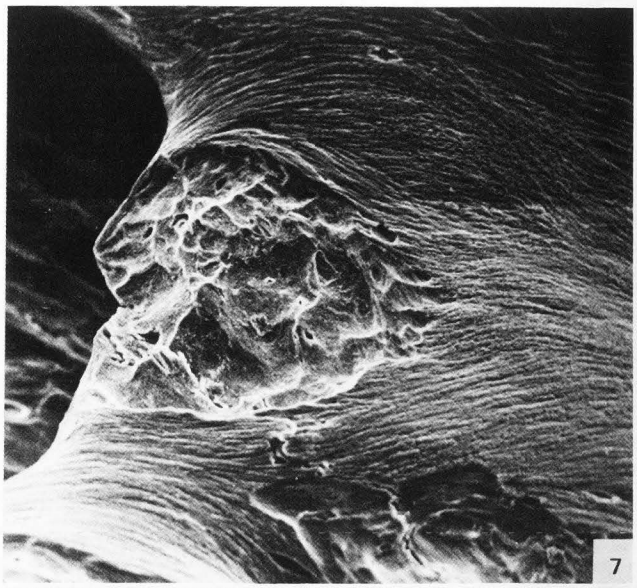
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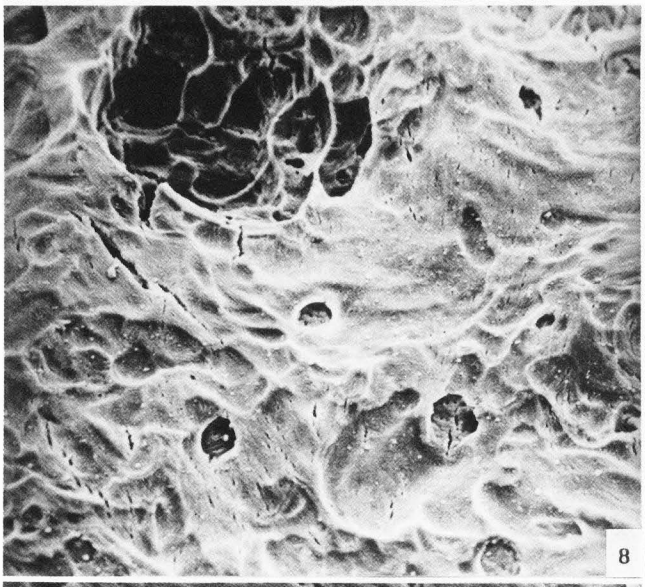
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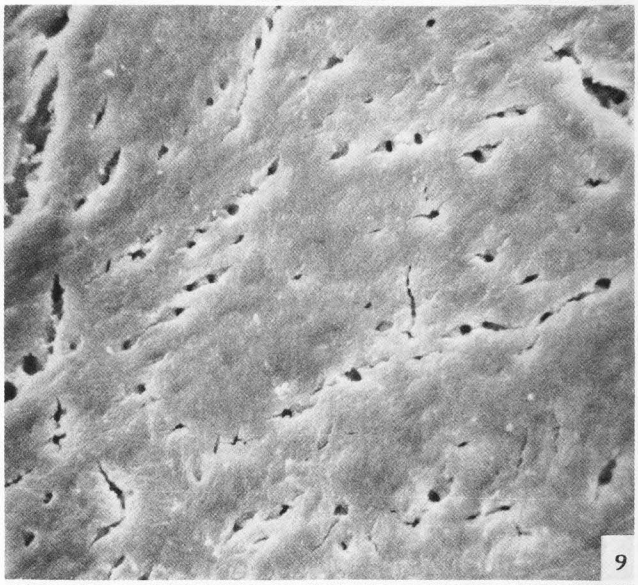
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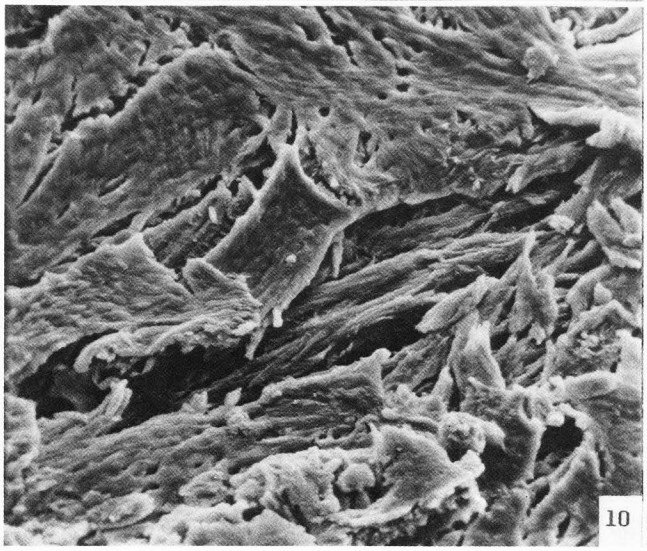
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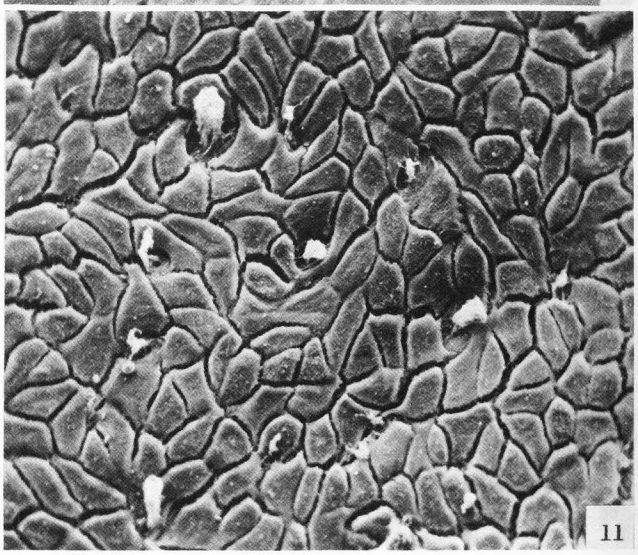
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Figure 6. Resorbed lamellar bone matrix surface, adult rabbit scapula covering cells removed by trypsin. Fieldwidth = 110 μm.

Figure 7. Human femur endosteal trabecular bone surface showing resorption into mineralised matrix. Some parts of the matrix were still mineralising as judged by the incomplete appearance of the collagen fibres in this anorganic preparation. Fieldwidth = 250 μm.

Figure 8. Endosteal surface of rat bone from animal given parathyroid extract 3 daily doses of Parathormone which stimulates resorption and mineralisation of free matrix surfaces. Fieldwidth = 176 μm.

Figure 9. Surface of bone matrix from similarly treated animal showing hypermineralisation of resting bone matrix surface. Fieldwidth = 17 μm.

Figure 10. Another area from similar specimen showing cleavage of well mineralised layer of matrix from less mineralised sub surfaces layer after the specimen has been made anorganic. Fieldwidth = 78 μm.

Figure 11. Tesselation of osteoblasts covering the endocranial surface of the rat calvarium. The small gaps between these cells are induced by CPD shrinkage. Fieldwidth = 176 μm.

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evidence has since been collected which supports the idea that osteoclasts or their precursors are transported via the blood stream: these studies, including the key experiments of G Gothlin and JLE Ericsson, and SC Marks and DG Walker are referenced and reviewed in ref. <22>. We interpret our SEM findings as possibly indicating that osteoclastic resorption stimulated by PTH occurred with the minimum requirement for migration of these cells.

Secondly, it was found that when compared with control animals, large areas of bone surface were converted to what we called the "prolonged, resting" condition, identified by the progression of mineralization beyond the limits of the superficial collagen fibrils and bundles of free bone surfaces; thus leaving a smooth, less detailed surface in anorganic preparation. In other words, PTH in the whole animal promoted mineralization of the bone matrix, particularly at its surface (Fig. 9).

In some instances, clear cases were identified where this surface mineralization overlay areas in which the osteoid had not mineralized fully. Upon making such specimens anorganic, patches of the superficial mineralized layer could be seen to break away (Fig. 10). This confirmed the impression that the PTH effect and/or combined with the expected hypercalcaemia, could result in a "dumping" of mineral from blood into the nearest available receptive location in bone, and this happened to be osteoid <21>.

The mineralization of superficial osteoid promoted by PTH in these experiments is, of course, abnormal and the wrong way round - osteoid normally mineralizes at its deep surface. However, such a mechanism would provide the means for allowing osteoclasts to "see" mineral at the free bone surface if these cells are dependent for their normal function upon the recognition of a calcified matrix.

Bone cells

The first SEM observations of bone cells were made in a convenient location where all other overlying tissue layers could be easily stripped away with apparently minimal disturbance to the osteoblast pavement <23>. Such a site is the endocranial surface of the skull cap (calvarium: Fig. 11). In young rats, most of this surface is formative, and is covered with a tesselation of cells <24> which are "cuboidal" and in close lateral contact prior to the induction of drying-associated, shrinkage artefacts <25>.

The shrinkage artefact problem provoked us into studying its origins, which we now more clearly understand <26, 27>. If we use the advanced drying techniques of freeze drying (FD) or critical point drying (CPD), we are left, at one near-final stage in either process, with a specimen with no free water, but some firmly-bound structural water. This stage occurs after the sublimation of ice in FD, and after the pressure is reduced after the critical point in CPD. In both cases, the drying of the **dry** sample induces separation of osteoblasts from each other. The well mineralized bone matrix hardly shrinks at all in comparison <25-27>.

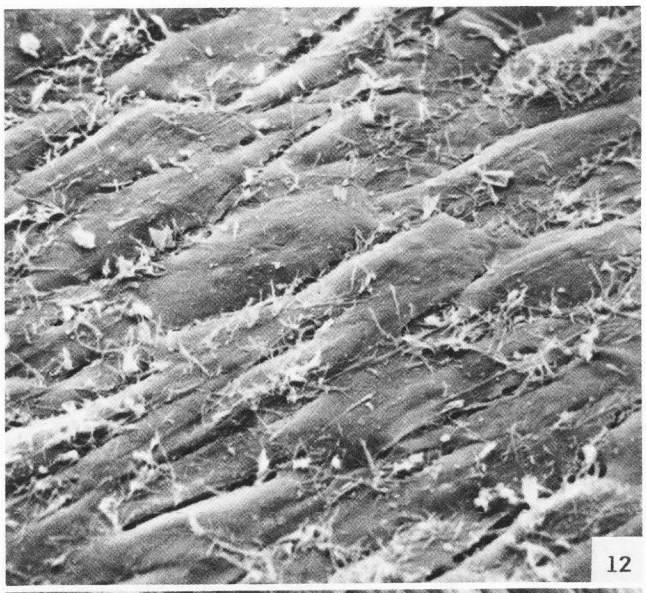
Measurements of the areas covered by osteoblasts showed that they fell within a closely defined range - the secretory territory of the osteoblast <22,23>. The same type of preparation was used to provide details of the normal morphological polarisation of osteoblasts <24>.

Hormone experiments on bone cells

After showing that bone cells in this system would survive well in culture conditions, the behaviour of these cells in response to physical and hormonal stimuli was investigated. These studies showed that the effect of the resorption inducing hormone, PTH, was primarily on the osteoblast <28-31>, and not on the osteoclast as had been believed up to that time. A direct response of osteoblasts to the vitamin D metabolite $1,25(OH)_2D3$ was also demonstrated in this way <28,29>.

These studies also showed that osteoblasts are actively migratory cells, spreading on to deliberately denuded portions of the bone, and on to surrogate substrates, such as pieces of glass or tooth placed in contact with the osteoblast layer or with bone marrow <30>: this tendency of bone cells to migrate has since been used as the basis of an important method for isolating bone cells in culture <32>.

Returning to the hormonal responsiveness of the bone cells as monitored by SEM <28-31>, osteoblasts showed no response to calcitonin (CT). The dorsal surface ruffling which is an adaptation shown by osteoblasts in culture was unchanged by addition of CT. However, osteoblasts responded dramatically to PTH in several different ways, which led to the suggestion that these are the cells which regulate bone resorption PTH induced the formation of gaps in the otherwise nearly continuous osteoblastic pavement, which, in the short term, were greater than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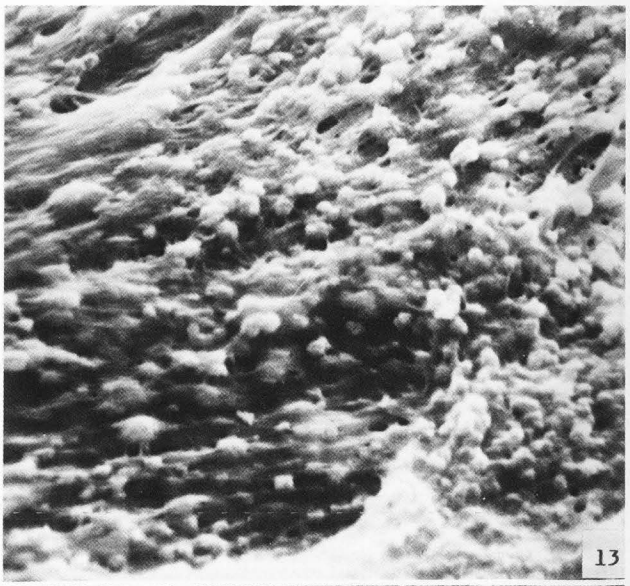
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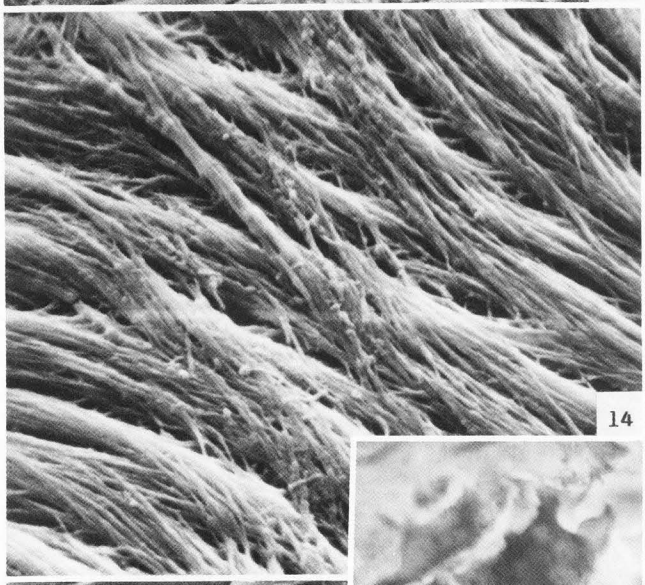
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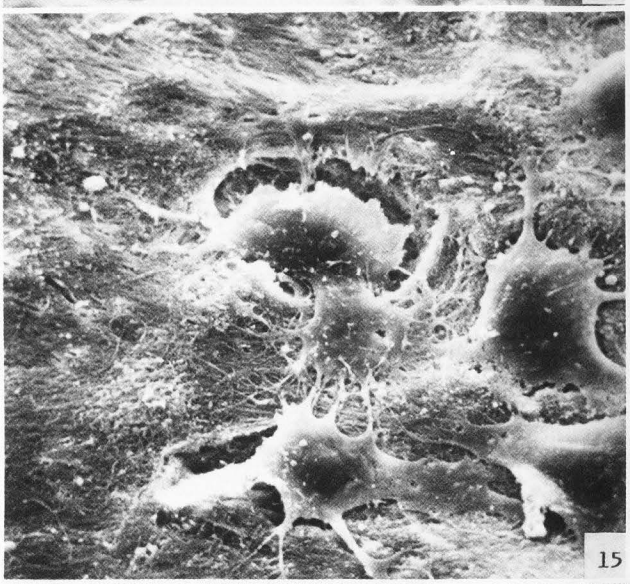
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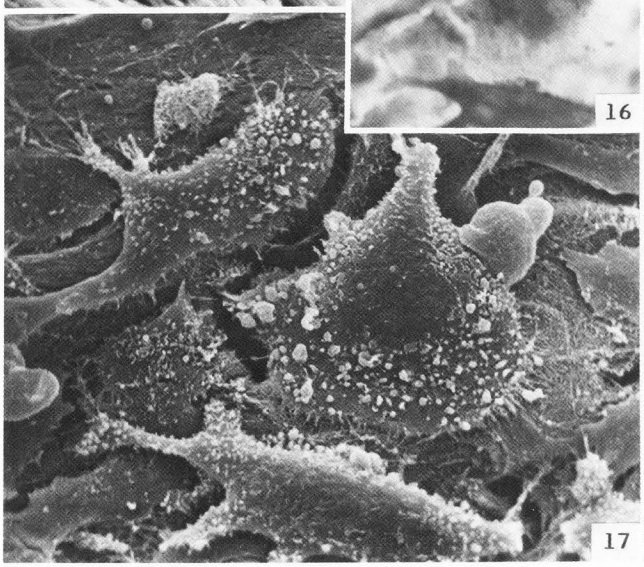
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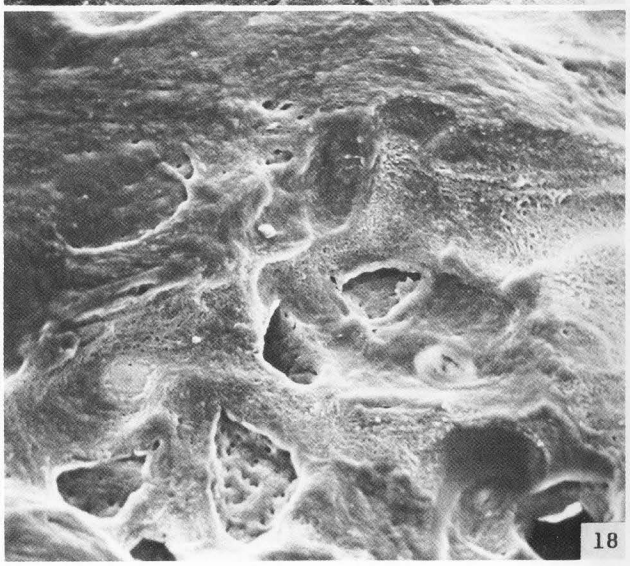
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Figure 12. Osteoblasts on endocranial surface of rat calvarium cultured with added PTH for 24 h. This induces marked cell shape changes. Fieldwidth = 100 μm.

Figure 13. Rat fetal bone treated with trypsin: Idea was to remove the osteoblasts but trypsin dissolves fetal osteoid. Thus this surface shows mineral clusters at the mineralising front, The overlying matrix having been removed, possibly by trypsin activation of latent collagenase. Fieldwidth = 22 μm.

Figure 14. Adult rabbit scapula near to area undergoing resorption: Matrix cleaned by trypsinization as in figure 13. Note that collagen fibrils are intact. Fieldwidth = 23 μm.

Figure 15. Osteocytes at endocranial surface of rat calvarium released by osteoclastic resorption migrating from their lacunae after 24 h in vitro. Fieldwidth = 48 μm.

Figure 16 (inset). Light micrograph of toluidine blue stained preparation of osteoclasts on rat calvarium. Fieldwidth = 92 μm.

Figure 17. Same field as figure 16 after preparing by CPD for SEM. Fieldwidth = 92 μm.

Figure 18. Same field as figs. 16 and 17 after removing the cells by treatment with NaOCl. Fieldwidth = 92 μm.

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those occurring in controls. Later, however, PTH caused an important change in osteoblast morphology and a reduction in the loss of cells in culture conditions so that they, as a whole, more effectively covered the bone surface. Part of the change in cell morphology was for the osteoblasts to elongate considerably, and to align themselves in parallel arrays - more parallel, in fact, than arrays of patches of similarly elongated control culture cells (Fig. 12). PTH treated cells were also able to migrate with apparently undiminished vigor.

Another very interesting finding was that many osteoblasts would go into mitosis following the removal of PTH containing medium and its replacement with control medium. This mitogenic activity of PTH, by leading to an increase in the potential number of bone forming cells, might be part of the explanation of the stimulatory effect of pulsed doses of PTH on bone formation which has been established clinically <33>.

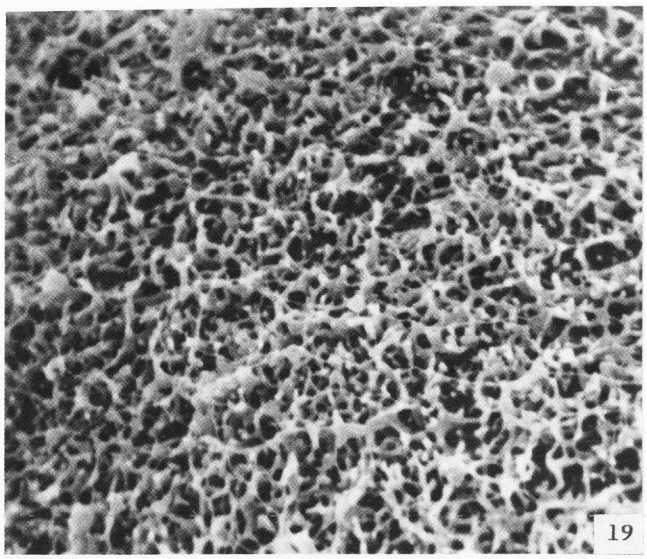
PTH influences on the cytoskeleton of cells of osteoblast-like lines have recently been documented in detail by Rodan and co-workers <34>.

Other than osteoclastic bone destruction mechanisms? and the fetal vs. adult bone problem

In searching for a method to remove osteoblasts from bone matrix surfaces, we tried to use trypsin at neutral pH, since this enzyme is widely used in cell culture technology to dissociate cells and to dislodge them from artificial substrates (Figs.13 and 14). This works well with the more mature forms of bone with oriented collagen, leaving the collagen fibres of the matrix nicely exposed to view (Fig. 14). However, in the case of fetal or woven bone (Fig. 13), we found that the osteoid itself was largely destroyed, leaving the mineralizing nodules or calcospherites of the mineralizing front exposed to view <10>. Trypsin is known not to affect collagen under these conditions, so what was the explanation? At the same time, Vaes <35> reported the presence of latent collagenase in fetal mouse bones which could be activated by various enzymes including trypsin. The idea that trypsin was acting in this way was given further weight in an experiment which showed that the destruction of avian fetal bone by trypsin was prevented by the action of the trypsin inhibitor cysteine <36>. This evidence provided morphological grounds supporting the view that the inactive pro-collagenase <37,38> might really be incorporated in fetal bone matrix as a kind of built-in, self-destruct mechanism. However, the SEM evidence so far has not shown that a similar mechanism exists in the mature form of bone. Recent work which purports to show the degradation of lamellar bone osteoid under the action of the osteoblasts has only shown the effects of the cell culture conditions used <39>. Because fibroblasts are able to participate in the degradation of the matrix which they have made, we cannot exclude the possibility that osteoblasts could undertake the same activity and remove osteoid in the preparation of bone matrix for osteoclastic degradation. We do not believe that, under some non-physiological circumstances, osteoblasts could not release matrix degradative enzymes. However, evidence for this occurring in normal resorption is lacking.

If osteoblasts were to be able to degrade non-mineralized bone matrix, why should not their derivatives, the osteocytes, do likewise? We have mentioned above, and reviewed previously <40> the reasons for believing that this does not occur.

Culture experiments also showed another character of osteocytes. Although encapsulated in solid, mineralized bone matrix until their rescue and liberation by osteoclastic resorption, osteocytes retain the ability to migrate (Fig. 15). Indeed, liberated ex-osteocytes are certainly a significant component of the singly nucleated cell population left on a resorbing surface, and they may be the "helper cells" <41>. However, although it cannot be excluded that osteocytes may produce enzymes which help to degrade the residual demineralized collagen fringe left on a resorbed bone surface, it should be noted that AR Ten Cate (personal communication) has seen no similar cells in resorbing kitten deciduous dentine. At least in the latter case, then, when no new reparative hard tissue formation need take place, no "helper" cells can be envisaged, and, of course, there were no osteocytes present to be released from the dentine. In this respect, we might propose that one role of osteoblastic "collagenase" may be to remove the residual demineralized matrix fringe after osteoclastic resorption. It is known that even in adult lamellar bone, the first layer of new bone deposited on an ex-resorbed surface, tends towards the fetal condition <42>. We hypothesize that "fetal" osteoblasts can liberate a matrix degradative enzyme: they may do this to ensure that a "clean start" is made by removing old, partly degraded matrix, or this may just occur fortuitously.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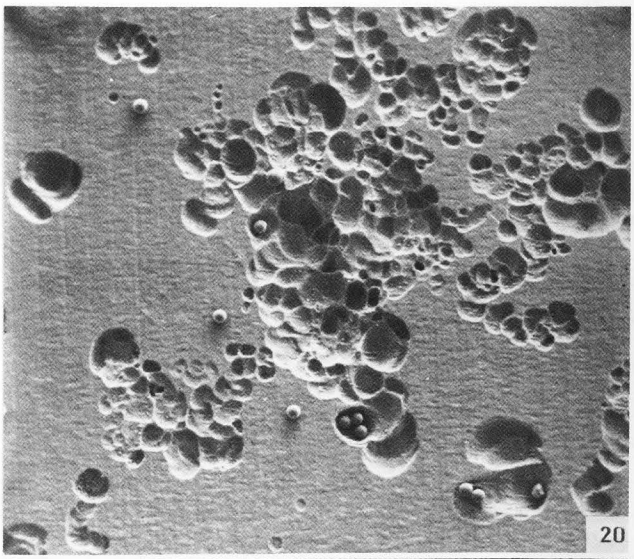
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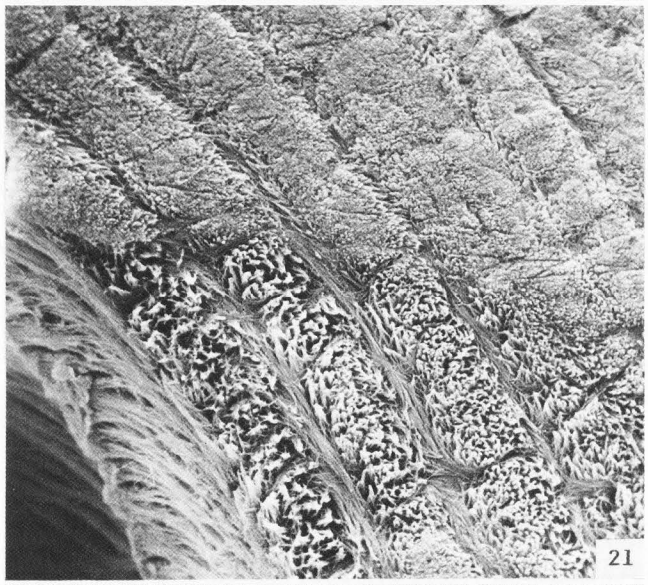
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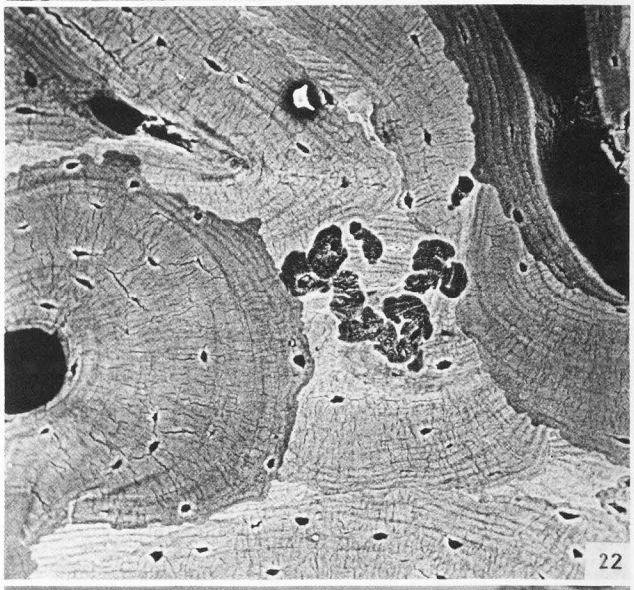
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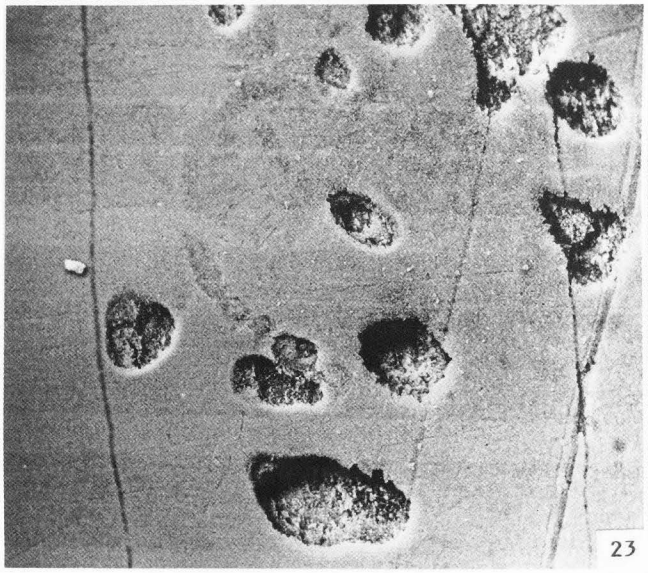
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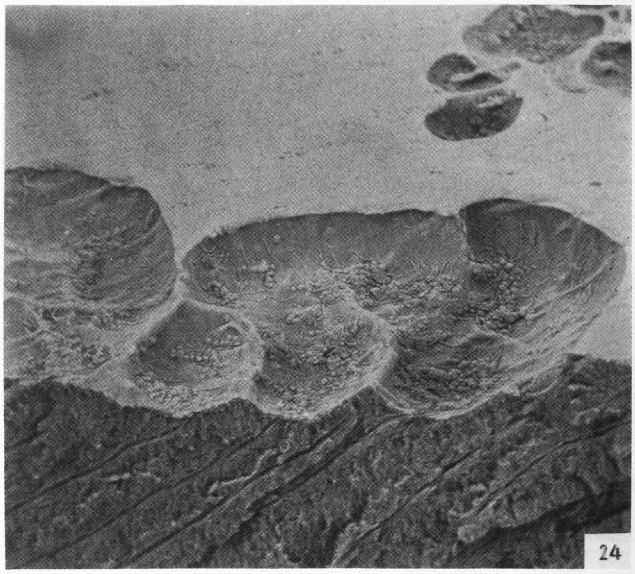
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Figure 19. Part of the ruffled border zone of an osteoclast peeled separated from bone. Fieldwidth = 18 μm.

Figure 20. Surface of slice of sperm whale dentine cultured with chick osteoclasts for 19 days, showing, extensive resorption which has occurred in this period. Note the wide range of size of the resorption pits. Some new osteoclasts may have differentiated from marrow cells in this period. Surface decorated with 10 micron polystyrene latex spheres. Fieldwidth = 480 μm.

Figure 21. Lower part of field shows edge of resorption bay in human compact bone made by chick osteoclast, revealing the alternate orientation of lamellae. Fieldwidth = 48 μm.

Figure 22. Chick osteoclastic resorption of human compact bone. This field is selected to show apparent selection of densely mineralised areas showing as white in this BSE image. However, careful study shows equal selection for all density phases in bone and unmineralised osteoid. Fieldwidth = 450 μm.

Figure 23. Internal surface of mollusc shell resorbed by chick osteoclasts. Fieldwidth = 104 μm.

Figure 24. Sperm whale dentine resorbed by osteoclasts originating from chick marrow in 19 day culture. Sample broken and tilted to show profile of fracture line through resorption lacuna. Fieldwidth = 90 μm.

The osteocytic osteolysis concept

From our own viewpoint, osteocytic osteolysis became an unsupportable concept when we were unable to discover degradative changes in the matrix surrounding osteocyte lacunae <6>. Other evidence discrediting this hypothesis was considered by AM Parfitt in his influential review <43>. Every case of "osteocytic osteolysis" so far described in the literature can be traced back to the failure to recognize the spectrum of bone and bone cell types from woven (fetal) to adult (lamellar). SEM studies clearly emphasize the points of difference in these tissue types: large cells incorporated in large numbers, often close to each other, with the mineralization of juxta-lacunar collagen often incomplete in the back wall of the forming osteocyte lacuna in the mineralizing front region, are the hallmarks of the rapidly forming, fetal bone type. Such characters are found in various bone diseases, particularly those in which bone turnover rate is increased. Thus irregularly mineralized lacunar walls can be found in all forms of rickets and hyperparathyroidism <40>, but they are all formed as such in the first instance. There is no established evidence for osteocytic involvement in bone matrix destruction or demineralization. The converse is not true however.

Rat osteocytes may make perilacunar matrix (Fig. 2) which mineralizes to a high degree. If they do not encourage the mineralization of old bone matrix, they certainly permit it to occur - as is demonstrated by the maturation (i.e. increase of mineralization) phenomenon found in all lamellar bone.

The origin of osteoclasts

A few years ago, it was fashionable to assume that osteoclasts arose by the fusion of mononuclear cells of the monocyte-macrophage series <44>. We used immunological marking techniques to examine this hypothesis, using the SEM as the means of observing whether rosettes of RBCs formed in the assay system. The results were negative - they demonstrated that osteoclasts did not bear the Fc or C3 receptors characteristic of all cells of the macrophage-monocyte lineage <45,46>, suggesting a separate origin at an earlier stage of stem cell differentiation. This finding was at first treated with considerable scepticism, but has now found acceptance following recent work which has extended and confirmed the original observations <47,48>.

The isolated osteoclast resorption assay <49>.

Most previous assays of the bone resorption process in vitro have been based on the measurement of biochemical parameters based upon all events affecting all parts of all the matrix and all the cells in a limited organ culture system <e.g. 50-53>. The measurement of the release of ^{45}Ca or ^{3}H proline from prelabelled bones is a sensitive approach: results are usually expressed as the ratio of experimental to control values <50-52>. The release of calcium can also be measured by simple chemical, colorimetric procedures <53>. The problems with all these methods are that they do not establish that the release of matrix or mineral components is the result of osteoclastic activity, or to what extent osteoclastic activity is controlled by osteoblasts or furthered by other cell types, for example, macrophages. Further, these assays always use fetal bone, and fetal bone matrix is degraded by non-specific proteases which may activate latent collagenase <10, 36>. Thus, as discussed earlier, osteoblasts may themselves be important in the degradative process in cultured fetal bone. Analysis of sections of bones prepared for light microscopy has also been used in resorption assays, but this poses a sampling and measurement problem in a rapidly changing tissue. We therefore wished to establish a system in which osteoclasts could be tested in isolation and on a known starting platform (Figs.20 - 24). The system has been successful and has been adopted by other groups <49, 54-61>.

The measurement of the volume of bone or dentine removed per resorptive episode in vivo might in itself be a useful parameter in characterizing this process under different normal and pathological circumstances, and this is where we began using SEM stereophotogrammetry <8, 14-19>. To provide the instrumental back-up for an assay of bone resorption based on the activity of individual cells, we developed computer aided systems for the measurement of the volume of resorption pits <8, 18, 19>. XYZ data acquisition from the RS3 instrument built for us by Ross Instruments <18> was controlled by a software package (OC3D) for a Sharp MZ80K microcomputer. This instrument has now been superceded by an

improved one called SFS3 and a new software package (STEREOSHIPS) written for a Sirius microcomputer <19, 49>.

In our earlier SEM studies of in vivo resorption we could assign accurate values to the volume, areas, radius of curvature, and depth of individual "scoops" <8>, but these were minimal values because such resorption almost invariably started in areas which had had curved surfaces. There was no reference plane against which to measure, and the edge of the resorption pits were frequently affected by later resorption in an adjacent "episode" (Figs. 16-19). These problems did not prevent us from finding large differences between the volumes of resorption pits in normal bone and normal deciduous dentine.

Cachalot dentine. In developing our in vitro, single cell, resorption assay system, we chose to use dentine as the surrogate-for-bone substrate. Further, we chose to use male sperm whale (in old English and modern French "cachalot") dentine. The reasons for these choices were that dentine is a more homogeneous material than bone, avoiding the pitfalls of variations in composition from one micro-area in the substrate to another. There are no holes in a cut piece of dentine through which our osteoclasts can fall (Fig. 20). There are plenty - the original Haversian canals (Figs.21 and 22) - in any piece of bone. Male sperm whale mandibular teeth are so large that we can tailor large numbers of large flat pieces from one tooth. We have many cells on one substrate, and the substrates have the same origin in many different experiments <19, 49, 54 - 58>.

What has the in vitro system shown?

Solo. The in vitro system shows that isolated osteoclasts, which can be observed to be well out of contact with other types of cells which settle on to SWD, bone or other surrogate substrata, are perfectly capable of resorbing all the phases present in these materials <54-63>. This type of experiment showed, therefore, that activated osteoclasts can dissolve all phases of bone (see also <3>). The absolute requirement for the involvement of osteoblasts, macrophages or other cell types can be excluded. These experiments also showed that osteoclasts in vitro may move away from a resorbed area of bone or dentine, leaving a "demineralized collagen fringe" in each lacuna. The depth of these fringes is apparently more extensive than we have encountered in studies of in vivo bone resorption using the SEM. Something in the resorptive process is then different in the in vitro system. Amongst various possibilities <63> are that the enzymes necessary to complete the degradation of bone matrix may disperse too easily in vitro, or be deficient in quantity relative to the amount of acid produced in vitro. They may also be produced by other cell types, such as osteoblasts, which are to lay down the reparative bone, or they may be of fibroblastic, macrophagic or other origin.

Bone. We have also used human bone samples in in vitro experiments, and compensated for our lack of knowledge about the variations in the composition of the substrate (Fig. 21) by determining this afterwards, primarily by the use of BSE A + B imaging of samples which were well polished before initial use (Fig. 22). Reid <11> has, in this way, been able to show that avian osteoclasts have no predisposition to resorb bone of different density due to different degrees of maturation-mineralization. Nevertheless, the local depth of resorption is affected by the degree of mineralization; just like the situation in tooth resorption (<1> & Fig. 1). Thus within any one resorption lacuna, the depth of destruction is less in the more mineralized bone phase <62>. This observation has validated our earlier decision that a more uniform substrate would be a wiser choice.

Other substrates. Our in vitro studies also showed that other mineral and other organic matrix substances could be tackled by isolated osteoclasts; and further, that mineral-alone and matrix-alone substrates are resorbed <58>. Considering the various materials we have examined: dental enamel, avian egg shells and molluscan shells (Fig. 23) contain no collagen, but have other specific proteins and protein-carbohydrate complexes. Avian egg shell and mollusc shells contain no phosphate: these are calcium carbonate (calcite or aragonite) containing materials. As would be expected from the higher acid solubility of carbonate compared to calcium phosphates (including hydroxyapatite), these shells are more prone to osteoclastic demineralization and can be used as sensitive markers to show where an osteoclast has migrated during an experimental period. Pure calcite and pure apatite synthetic substrates were also attacked, showing that no matrix need be present. Further, we have shown that demineralized dentine and bone and as yet unmineralized predentine and osteoid are also resorbed - though with an evident reduction in incidence compared with the mineralized matrices.

Hormones. Our in vitro studies have shown that calcitonin (CT), the hormone which suppresses bone resorption in vivo and osteoclastic motility in vitro, does not stop resorption by osteoclasts in culture at the exact doses which eliminate peripheral cytoplasmic movement on plastic <55,56>. This result shows that demonstration of cessation of movement is not necessarily a demonstration of cessation of resorption. Levels of resorption are, however, measurably reduced and at higher concentrations may be suppressed entirely; there is an obvious correlation between movement, the cytoskeleton, and exocytosis. Resorption also occurs in serum-free medium, without any added hormones.

Longevity. More recent experiments have shown that osteoclasts originating both from the minced - chopped bone fraction and the marrow-only fraction of neonatal long bones of chicks are able to survive and to continue to be able to resorb for periods of at least 6 weeks <63>. This longevity in culture provides an estimate which is larger than most previous deductions <64-66>. However, we consider it likely that precursor cells differentiated to become osteoclasts within this experimental period <63>. A proportion of the resorption lacunae found during only 3 week

culture periods (Figs. 20 & 24) are exceptionally large, single and smooth, and therefore presumably single-cell origin scoops formed by the continued activity of large osteoclasts. Some have been shown to have volumes over 300 pl, hundreds of times greater than those found within 24 and 48 h periods <49, 63>. In parallel observations made by LM, we have shown that there is a small population of osteoclasts with exceptionally high numbers of nuclei in the starting population. These may presumably be added to by the fusion of others during the experimental period.

The actual numbers. Measurements of volumes of SWD resorbed by, for example, chick osteoclasts indicate mean rates per cell with an average of 8 nuclei of 2200 fl/day over shorter time periods <49>. There are good correlations (R greater than 0.96) between volume and area of resorption pits. However, the slope of the regression line for area/volume is different at different time periods and for osteoclasts originating from different species. It is therefore unsafe to assume that volumetric (3-D) measurement can be dispensed with in studies of this type, or that rigorous methods can be short cut <67>.

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Discussion with Reviewers

Reviewer I: What contribution could the SEM make towards resolving some of the outstanding issues, such as resorption-formation coupling and the origin of the osteoclasts?

Authors: The SEM is just one tool which can be used to focus on small facets of the bone resorption problem. As regards resorption formation coupling, we have indicated that it enabled us to see that this was happening in the extra-skeletal context of cellular cementum formation on the roots of human permanent teeth. It is improbable that the necessary global view would have been obtained by reconstructions from serial sections from any other microscopic method. The survey potential of the SEM has not yet been exploited in examining skeletal resorption-formation coupling at the local scale.

As regards the origin of osteoclasts, the SEM provided the means to observe that the surface antigens of the cells of the monocyte macrophage series were not borne by osteoclasts. At that time it was widely assumed that the cell types were at least very closely related. So, at least, even if the SEM cannot solve problems, it can raise them.

However, we believe that the SEM has a role to play in investigating the function of osteoclasts. An example of this is its sensitivity to the detection of changes in the surface of a substrate using BSE imaging. This may be very important when testing the resorptive ability of putative osteoclast precursor cells, since it is a characteristic ability of an osteoclast to demineralize the tissue it contacts.

Reviewer II: It is not justified to extrapolate from the standpoint that lacunar margin modification by osteoclasts does not exist, to conclude that their precursors, the osteoblasts, cannot remove osteoid. The authors have overlooked recent evidence for an osteocyte-specific cell surface antigen (PJ Nijweide & RJP Mulder, A monoclonal antibody specifically directed against osteocytes. Calc. Tiss. Int. **38 suppl**, Abst. 28, 1985).

Authors: Other connective tissue cells are able to dismantle the matrix which they have made. This is amply illustrated in many studies on the activities of fibroblasts (as an EM based example, see AR Ten Cate (1972) Morphological studies of fibrocytes in connective tissues undergoing rapid remodelling. J. Anat. 112: 410-414). Our argument is that evidence for osteocytes or osteoblasts performing this function during normal resorption in vivo is lacking.

Lacunar margin modification by osteocytes does occur, but this has only been demonstrated in the sense of the accretion of new matrix and mineral in the walls of osteocyte lacunae. Perilacunar bone was not discovered by SEM - that can be attributed to the work of IA Mjor (Bone matrix adjacent to lacunae and canaliculi. Anat Rec. **144**, 327-329, 1962) using the TEM. However, it became very much clearer from the earliest SEM studies that perilacunar bone was an interesting material which can be deposited by osteocytes in some locations and/or in some species.

It remains to be discovered what is the nature of the antigen discovered by Nijweide's antibody to something at the cell surface of chick osteocytes. It is as likely that this represents new synthetic activity (something to do with perilacunar bone?), as that it may represent matrix degradative activity.

The examples we have chosen here from the study of hard tissue resorption, we state again, are essentially chosen to illustrate the merits of the SEM in this sphere of investigation. Of course, it has also been of great utility in studying the formation and the structure of the hard tissues, including many aspects of mechanical and clinical interference with these tissues in orthopaedics and dentistry.

O. Johari: In stereo-pair (Fig. 4), how can stereo-effect be observed?

Authors: A few persons can detect the stereo effect without the aid of any stereo-viewing device. For others, we suggest that the page be rotated 90° and viewed under a stereo-viewer.