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Effect of mineral content of human bone on in vitro resorption

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Summary. Osteoclasts, mechanically isolated from chick long bones, were grown in vitro on slices of human rib and femur. Evidence of their activity was assessed by secondary electron and backscattered electron (BSE) imaging in the SEM. BSE imaging was also used to study the relative degree of mineralisation of the bone matrix in which resorption had taken place. All bone phases were resorbed, from osteoid through to densely mineralised interstitial bone and reversal (cement) lines. Resorbing osteoclasts crossed reversal lines between osteons of different mineral density and moved both from higher to lower and lower to higher density phases. Where single loci spanned reversal lines, and thus breached bone of two different mineral densities, depth of demineralisation was inversely related to mineral density. The presence of an annular zone around some resorption loci, which may be caused by demineralisation beneath the osteoclast clear zone, was confirmed. Also, BSE imaging of polished substrata showed that significantly more osteoclastic activity had occurred at their surfaces than was apparent from the amount of cavitation present.

Key words: Osteoclasts – Resorption – In vitro – Mineral density – Osteoid

Introduction

The recent development of an in vitro assay in which the activity of single osteoclasts upon especially prepared substrata is examined in the scanning electron microscope (SEM) (Boyde et al. 1983, 1984; Chambers et al. 1984a) has opened the way for many questions to be addressed concerning the interactions of these cells with the tissues upon which they are active (Jones et al. 1985). One such question is the supposed requirement by osteoclasts of a mineralised matrix for the expression of their activity (Aaron 1981; Chambers et al. 1984b; Heersche 1978). A second concerns whether osteoclasts select older, more densely mineralised bone fractions or, alternatively, reject newly deposited bone during resorption (Tappen 1977). This idea pervades the bone literature relating to Haversian remodelling and is based on the premise that resorption occurs to replace dead bone or bone damaged during functional loading (see Currey 1984; Ham and Cormack 1979). A further notion is that bone which becomes very densely mineralised is less resorbable and leaves the remodelling pool.

These questions have been examined in the present study, following pilot studies which demonstrated the feasibility of the method used (S.J. Jones, unpublished work). Chick osteoclasts were grown in culture on human bone slices which included osteoid seams. The SEM was then used to analyse both the extent of resorption of these substrata and the relative mineral content of the bone in which each episode of that activity had taken place. The latter was possible using the backscattered electron (BSE) imaging mode (Boyde and Jones 1983a, b). Stereophotogrammetry was used to measure the extent of resorption in bone of different mineral density (Boyde and Jones 1979; Howell and Boyde 1984).

Materials and methods

Osteoclasts

Osteoclasts were prepared by mechanical separation (Chambers and Magnus 1982) from the tibiae and femora of pre-hatch chicks on the 19th day of incubation. Bones were dissected free of muscle and periosteum; the epiphyses were cut off and the diaphyses placed into Dulbecco's phosphate-buffered saline (PBS) and finely chopped with a scalpel blade. The PBS containing cells and bone fragments was then repeatedly sucked up and down in a wide-bore syringe to increase the dissociation of cells from bone. After a 30-s settling period, during which large bone fragments fell to the bottom of the dish, the cell suspension was aliquoted into multiwell dishes (Linbro) containing prepared substrata.

Substrata

Substrata were prepared from human ribs and from a human femur. The rib specimens were from sixth ribs taken at post-mortem from neonate, juvenile and adult subjects who had died suddenly and for whom the post-mortem report gave no indication of bone pathology. These bones were stored in 70% ethanol until used. Transverse sections, 200–300 μm thick, were cut at the junction of the anterior and middle thirds of the bone, using a water-cooled rotary saw fitted with a diamond-edged wafering blade. These slices were repeatedly ultrasonicated in singly, and then doubly, distilled water and stored frozen in the latter until used.

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Femoral substrata were prepared from the midshaft of an adult femur taken from an osteological collection. Prior to section cutting, the surface of the piece of bone to be cut was first ground on graded carborundum papers and then polished on a high-speed lapping wheel, using water-based 6-μm and 1-μm diamond compounds. Between each step in this procedure the block being polished was ultrasonicated in distilled water to prevent contamination of subsequent polishing beds. When polishing was completed, transverse and longitudinal sections were cut, ultrasonicated and stored as described above. The purpose of polishing was to produce substrata with minimal surface topography. This is an important requirement if successful analysis of mineral densities in bone is to be conducted by BSE imaging in the SEM (Boyde 1984).

Culture conditions

Substrata were left covered with the PBS containing bone and marrow cells for 30 min at 37° C in air to allow cells to settle on to them. They were then washed in fresh PBS, to free them of non-adherent cells and bone fragments, and placed into clean wells containing MEM medium with Earle's salts (Gibco), 10% heat-inactivated foetal calf serum, 100 IU. ml^{-1} penicillin and 100 μg. ml^{-1} streptomycin. Cultures were maintained at 37° C in a humidified atmosphere containing 5% CO_2 for between 24 and 120 h. Medium was replaced after 48 or 72 h.

Scanning electron microscopy

Cultures were terminated either by transfer of the bone slices directly into 3% glutaraldehyde buffered with 0.15 M cacodylate at 37° C, or by transfer to, and ultrasonication in, distilled water (to lyse and dislodge cells) followed by fixation. After 24 h fixation, they were either dehydrated through graded alcohols and then air-dried in a saturated atmosphere of Freon 113 (Boyde and Maconnachie 1984) or critical point dried from liquid CO_2, or freeze-dried following washing in distilled water and cryoprotection in a solution of chloroform in distilled water (Boyde and Wood 1969). Dried specimens were mounted on stubs using graphite paste and lightly sputtered with silver or gold. They were examined in a Cambridge Instruments Limited S4–10 SEM fitted with an annular configuration solid-state BSE detector (KE Developments, Toft, Cambridge, UK). The microscope was operated at 10 kV for secondary electron (SE) mode imaging and at 10 or 20 kV for BSE mode imaging. BSE imaging at 10 kV was used for the examination of cells at normal beam incidence, while 20 kV was used for density analysis.

Following examination in the SEM, some specimens were further treated to remove cells or organic matrix or both. Gold and silver coatings were removed from their surfaces by immersion in a dilute NaCN solution (Sela and Boyde 1977). After thorough washing with distilled water, they were placed into a 7% (50% of stock concentration) sodium hypochlorite solution for 15 min to make them superficially anorganic, or into a 0.25% trypsin-EDTA solution (Sigma) at 37° C for 2 h and periodically ultrasonicated. This latter treatment caused dislodgement of cells but did not (completely) remove the fine collagen fibrils lining resorption loci. Specimens were dried, mounted and coated as before.

Stereophotogrammetry

Stereopair micrographs of resorbed areas were recorded with a tilt angle difference of 10° (Howell and Boyde 1984). The floors of such areas were profiled using an SFS3 Stereocomparator (Ross Instruments Ltd., Salisbury, UK), which outputs digitised X, Y coordinate and parallax data to a microcomputer (A. Boyde, P.G.T. Howell and F. Franc, unpublished work).

Results

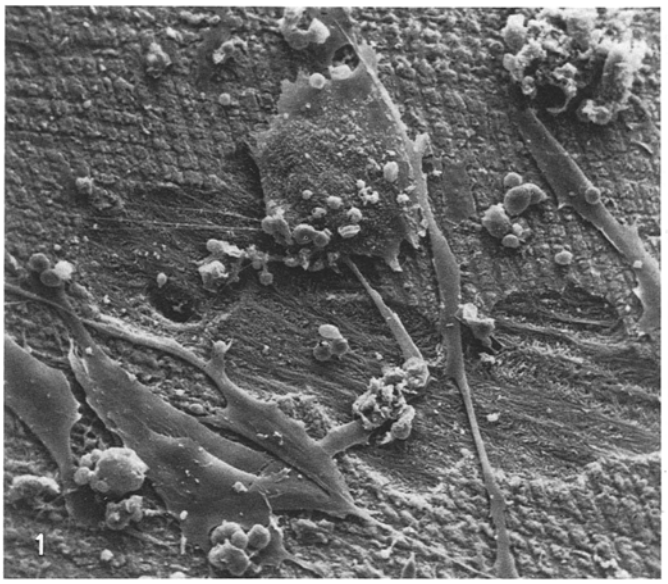
Resorption was identified on all the bone slices cultured with osteoclasts (Fig. 1). After 24 h in culture, resorption was evident as single scoops or as small complexes of conjoined loci. Individual loci varied in diameter from less than 5 μm to more than 35 μm at this time. With increasing culture duration, the size (and on subjective, rather than statistical, assessment, the number) of resorption complexes increased and after 72 and 96 h complexes composed of hundreds of loci were seen. There was also an increase in maximum resorption locus diameter with incubation time, loci with diameters as great as 100 μm being recorded after 72 h. All cultures showed a tremendous variability in the plan areas of resorption complexes and in the area and depth of individual loci within them.

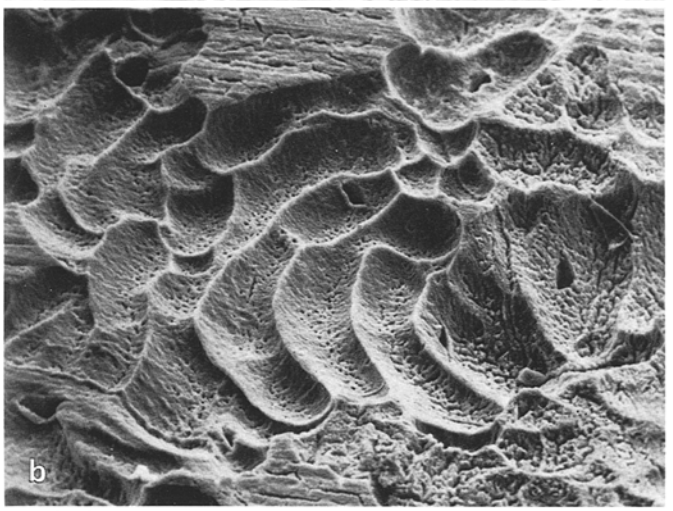
Single, isolated loci consistently had a circular or oval outline. However, within the large complexes seen after prolonged incubations, an elongated kidney shape was the predominant locus morphology and many such loci often abutted against one another in rows (Figs. 2, 3). The bases of resorption loci were always lined by a mat of fine, demineralised collagen fibrils, which could be removed by making the specimen anorganic. This mat was very sensitive to the tissue processing routines employed: using freeze-dried material it was shown that the length of demineralised collagen fibres at this site was much greater than initial results from critical-point dried material had indicated (Fig. 4). Also, this mat degraded during prolonged (several weeks) storage in 70% ethanol.

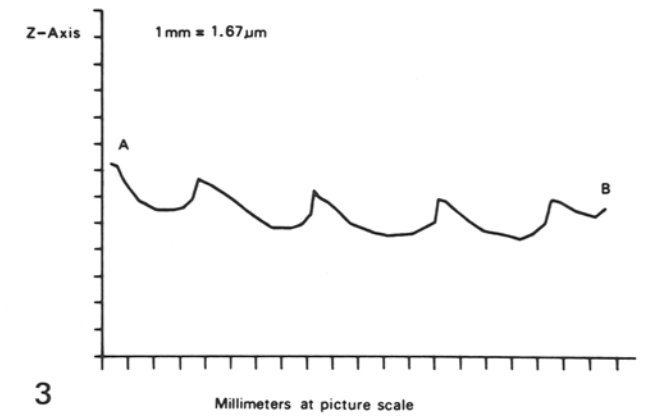
Fig. 1. Chick osteoclast (large cell, top centre) cultured for 72 h on the cut surface of a human bone slab. The shallow resorption complex with which it is associated shows the lamellar organisation of bone collagen and an opened osteocyte lacuna in its floor. Other cell types are present on and near the resorbed area. Fieldwidth 170 μm

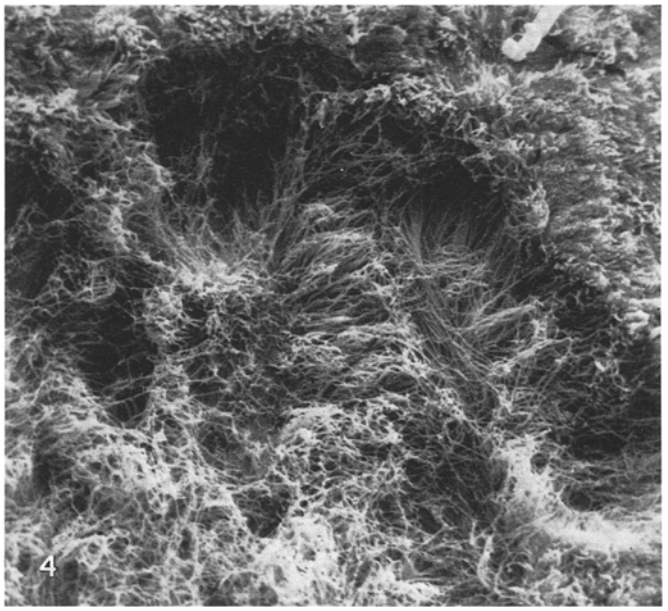
Fig. 2a, b. Stereopair showing part of a large complex of resorption formed in vitro on a slice of adult human rib cultured for 96 h with chick osteoclasts. The undercutting beneath the convex aspect of each of the abutting, elongated kidney-shaped loci at the centre supports the interpretation, derived from the plan shape of the loci, that the cell forming these loci moved from right to left. *Line AB* indicates the position of the stereophotogrammetric profile shown in Fig. 3. Anorganic preparation. Rotate 90° anti-clockwise to view. Fieldwidth 180 μm

Fig. 3. Profile across the floor of the resorption complex shown in Fig. 2









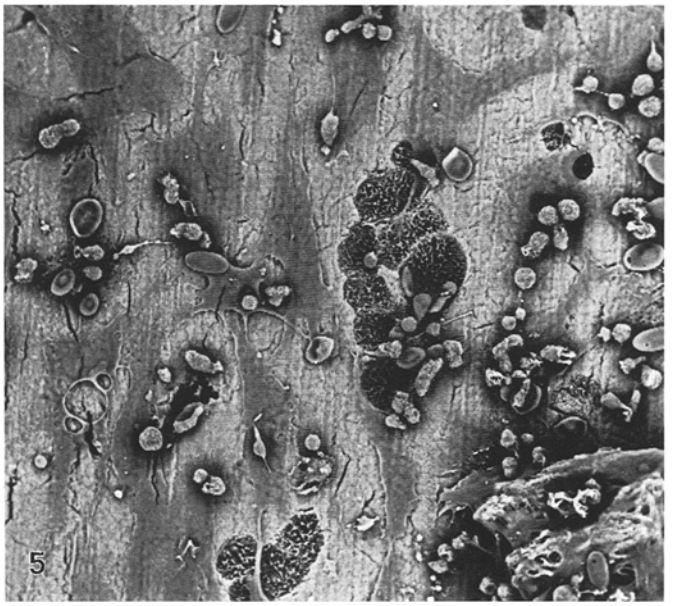


Fig. 4. Part of a resorption complex on a slab of human femur, which was fixed and freeze dried after being cultured for 72 h with chick osteoclasts. The extent of the demineralised collagen fringe in its floor is much greater than was originally thought from the examination of critical point dried specimens. Fieldwidth 45 μm

Fig. 5. A 10 kV backscattered electron (BSE) image, showing a small resorption complex which is aligned with cutting grooves on a rib substratum cultured for 24 h with chick osteoclasts. The osteoclast is no longer at the site of resorption. Other cells, seen as mid-grey shadows in this image, are also aligned with these grooves. Fieldwidth 165 μm

The rib sections had a grooved surface morphology, the surfaces having been left as cut with the diamond wafering blade. On these substrata, resorption sometimes showed alignment with the direction of grooving (Fig. 5). This was particularly evident in the case of small complexes seen after 24 h. Spindle-shaped cells (a mixture including fibroblasts and osteoblasts and their precursors) present in the culture system also showed considerable alignment with these grooves after 24 h. Neither were the femoral substrata completely topography free, for some was introduced by the polishing process itself. This topography was related to the density and structure of the substratum; for instance, denser bone phases polished more slowly than less dense phases. Also, collagen lying in the plane of the substrate surface polished more rapidly than collagen oriented normal to that plane so that lamellae were "etched out". Fibroblasts and osteoblasts sometimes aligned with this topography.

Osteoid

The examination of rib substrata revealed that chick osteoclasts had resorbed osteoid, as well as mineralised bone matrix, in neonate, juvenile and adult bone. This was confirmed by making specimens anorganic after they had been examined and checking that tissue interpreted as being osteoid (because of its quite different textural morphology and low BSE signal) was removed by this treatment. Osteoid resorption sometimes occurred as part of a complex which crossed a mineralised matrix-osteoid interface. However, it also occurred in isolation in osteoid, either as single loci or as small complexes (Fig. 6).

Resorption of different mineral density phases

BSE imaging allowed the mapping of mineral density phases in the polished femoral slices. In these images, resorption loci appeared dark against the surrounding, undisturbed bone surface (Fig. 7). In fact, it was easier to locate resorption in BSE images than in SE images, even if the substitute substratum had not been polished.

All mineralised phases, ranging from bone of low mineral density in partially completed osteons to densely mineralised interstitial bone, were resorbed. Analysis of 250 small resorption complexes (which could reasonably be attributed to the activity of a single cell) showed that 60% were confined to a single density phase and 14% spanned a reversal (cement) line and involved resorption of bone of different mineral densities on either side of the line (Fig. 8). The remaining 26% were divided into two groups: 19% had one or more loci situated against or directly over a reversal line, but never completely crossed it, and had two "tails" of resorption leading back on the same side of the line (Fig. 9); and 7% were within a single density phase, except for a single locus at one end of the complex, which was over or against a reversal line.

Two simple experiments were conducted in an attempt to test whether the distribution of resorption with respect to reversal lines differed from that which might have occurred by chance. In the first, one of the bone slabs used for the above analysis was montaged photographically using BSE imaging in the SEM (total area, 30 mm^2; magnified $\times 56$). Radial lines were drawn at every 22.5°, starting from a point near to the centre of the montage. By counting the number of reversal lines crossed by all 16 lines and measuring their total length (minus the length covering Haversian canals), 132 μm was calculated to be the average distance between reversal lines. An estimate of the average "length" of a resorption complex was calculated by measuring between the two most distant points of 80 (single cell) complexes from the screen of the SEM ($\times 225$ magnification; BSE mode; normal beam incidence); the result was 57 μm. In this case, the probability that a resorption complex would occur between two reversal lines would be $1-(57/132)=0.56$ (i.e. 56%). Using an average of 20 μm as the diameter of a single resorption locus, 15% of the remaining 44% of complexes would have a single locus over, or against, a reversal line and 29% would have more than one locus on both sides of the line.

For the second experiment, all single cell resorption complexes on each of the photographs in the above montage were drawn onto tracing paper (a total of 164 complexes). Each tracing was then laid over all the other photographs in the montage and the distribution of traced resorption complexes with respect to reversal lines in the photographs was recorded. In this case, 61.5% of resorption occurred within a single density phase, 18.5% of complexes crossed reversal lines, and 20% ended over a reversal line but did not appear to truly cross it. The results of these tests, and of the original experiment, are summarised in Table 1. The second test is likely to have generated a better result, since it considered resorption as covering an area, rather than attributing to it a single dimension, and allowed osteoclasts to approach reversal lines at any angle, rather than at normal incidence. It is interesting that both tests produced a value for resorption in a single density phase that was close to that obtained in the original experiment. This confirms that osteoclast seeding in this system is indeed random.

In a further assessment, each of the photographs in the montage was analysed using a stereological point counting grid. The bone under each point in the grid was scored according to whether it had a high, medium or low mineral density. The number of single cell resorption complexes in each of these density categories was also recorded from these photographs. Finally, the plan area of all resorption complexes counted was measured directly in the SEM. The specimen was examined at normal beam incidence in the BSE mode. Measurements were made at $\times 1{,}100$ magnification, using a point counting grid placed over the display screen of the microscope. The results of this analysis are shown in Table 2. The number of resorption complexes which occurred in the three density phases correlated directly with the frequency of these phases at the specimen surface. The mean plan area of individual complexes increased with mineral density, but this trend was not statistically significant.

In instances where osteoclasts had resorbed two adjacent, and different, mineral density phases, an attempt was made to determine the direction in which these cells had crossed reversal lines from the morphology of the resorption bays they had made. Kidney-shaped loci are formed when an osteoclast has resorbed once and then moves and is active in an immediately adjacent area (Jones et al. 1985). Thus, a cell forming such loci must have moved in a direction away from the concave aspect of the kidney outline towards the convex aspect (Fig. 2). Profiles across the floors of such loci (Fig. 3) and, also, our knowledge of the morphology of the leading lamella of motile cells (Abercrombie

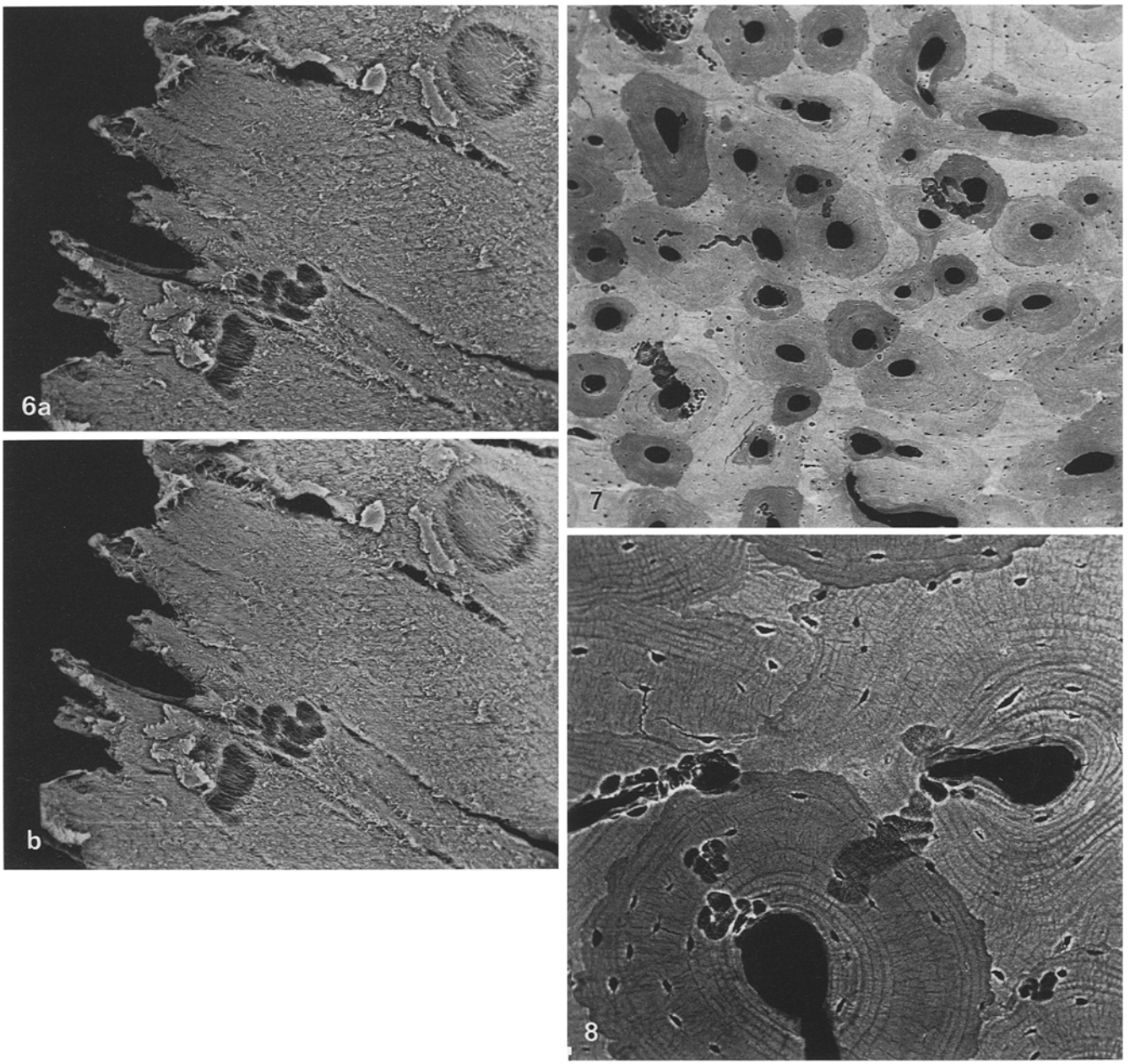


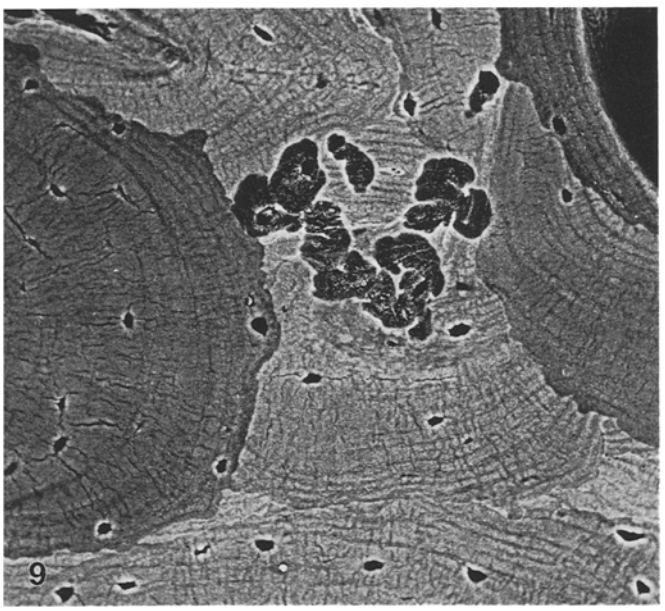
Fig. 6a, b. A 10 kV BSE stereopair, showing resorption of osteoid. At *bottom left* there is a small complex of resorption loci and at *top right* a ring of matrix degradation, which probably represents a site at which an osteoclast was initiating resorption as the culture was terminated. Note the fine fibrillar texture of the cut osteoid surface, which is not a feature of cut mineralised bone surfaces. The width of the osteoid was fortuitous and due to obliquity in the plane of section; 96-h culture of chick osteoclasts on an adult human rib slice. Rotate 90° anti-clockwise to view. Fieldwidth 95 μm

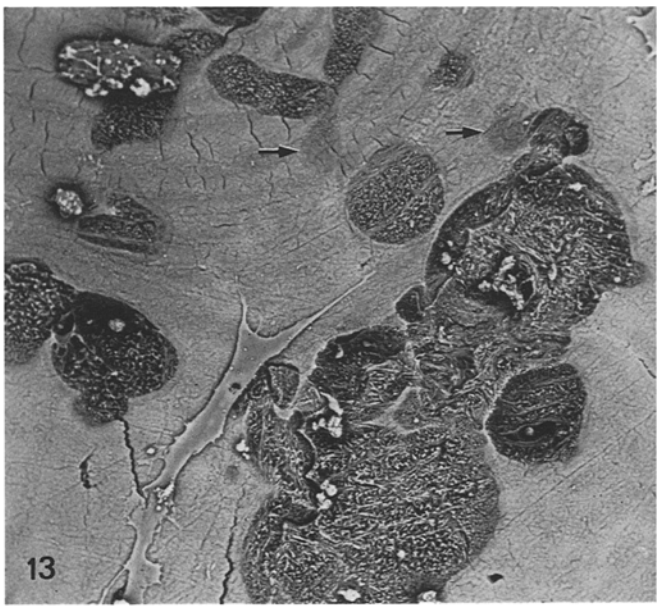
Fig. 7. A 20 kV BSE image of a polished femoral slice which was cultured for 96 h with chick osteoclasts. The image shows the relative mineral densities of different areas of the substratum and, also, snake-like tracks of resorption, which appear dark. Fieldwidth 1.7 mm

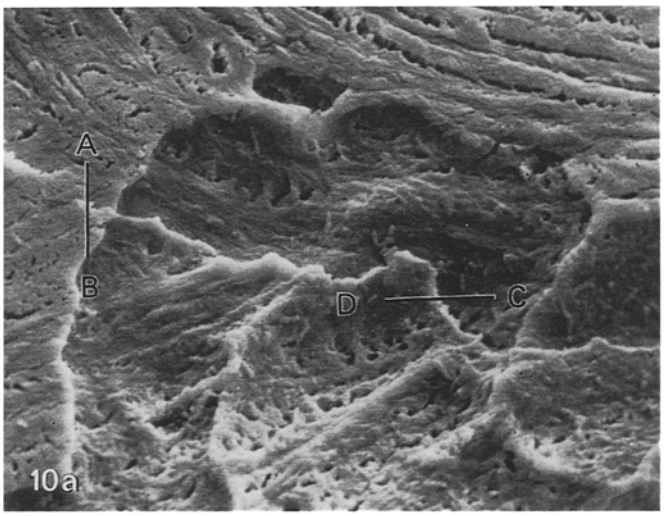
Fig. 8. A 20 kV BSE image, showing an osteoclast track across a reversal line. The resorption locus morphology indicates that the cell travelled downward from right to left, i.e. it has moved onto less densely mineralised bone and maintained its activity; 96-h culture of chick osteoclasts on human femoral slice. Fieldwidth 410 μm

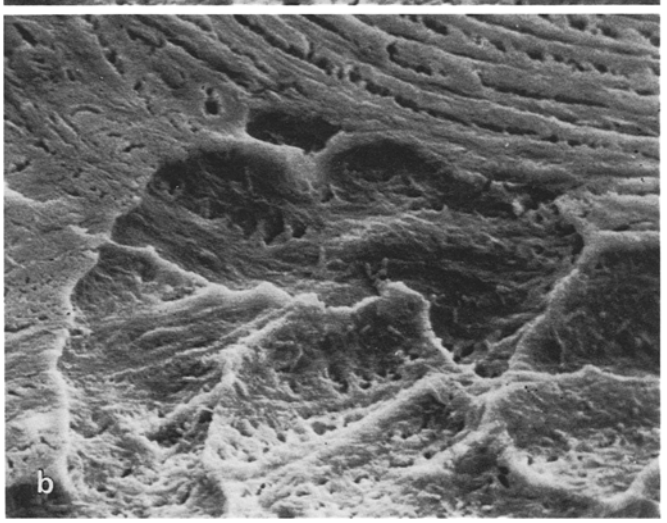
1982; Enteneur and Schliwa 1984) support this interpretation of the direction of osteoclast travel at such sites. On the basis of this interpretation, it was demonstrated that approximately the same number of cells had started resorbing in high-density bone and then moved to bone with a lower mineral content as had travelled in the opposite direction.

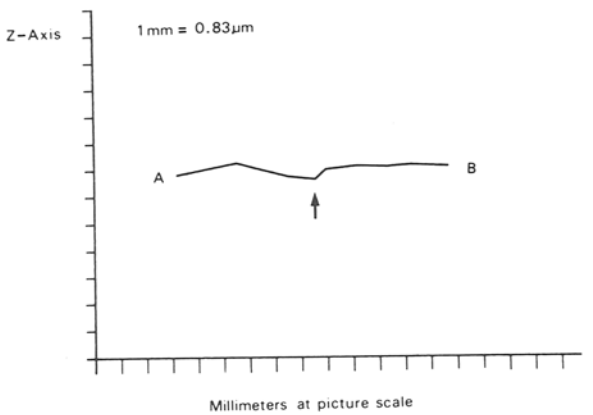
In a number of instances where a single resorption locus spanned a reversal line, BSE images were recorded to show the mineral density on each side of the line. These specimens











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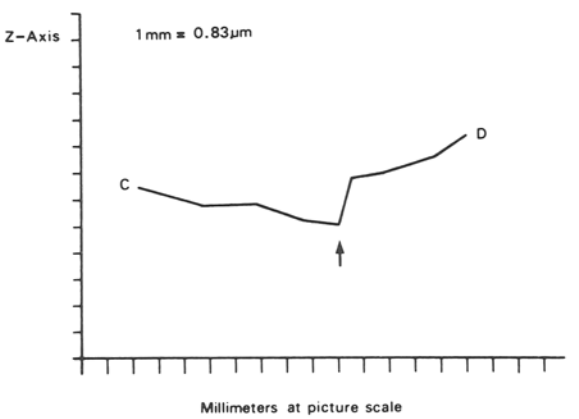

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Fig. 9. A 20 kV BSE image showing a resorption complex, or, perhaps, two separate complexes, with loci against, or over, two reversal lines. The osteoclast(s) may have failed to completely cross them as a result of interactions with osteoblasts or fibroblasts aligned with local lamellar relief, or the turns may be a chance occurrence; 96-h culture of chick osteoclasts on adult human femur slab. Fieldwidth 335 µm

Table 1. Distribution of resorption complexes with respect to reversal lines separating bone packets of different mineral density

	Original experiment	Test 1	Test 2
Resorption in single density phase (%)	60	56	61.5
Resorption against reversal line (%)	26	15	20
Resorption crossing reversal line (%)	14	29	18.5

Table 2. Distribution of resorption complexes with respect to mineral density phases on a human femoral substratum. The differences between the means in column 4 were not significant by Student's *t*-test. The large standard deviations shown in this column emphasise the variability in the plan area of complexes attributable to single cells

Bone density	% Area of specimen[a]	% No. resorption complexes ($n=117$)	Mean plan area of resorption complexes (μm^2) SD bracketed
High	22	25	913 (845)
Medium	48	44	676 (670)
Low	30	31	598 (744)

Total grid intersections counted = 1064

[a] Area analysed = 30 mm^2

were then made anorganic and re-examined in the SE mode. Stereopair photographs demonstrated that the (mineral) floors of such loci extended further into the bone on the less densely mineralised side of reversal lines and that the lines themselves (hypermineralised with respect to the immediately adjacent bone) stood proud of the bone on either side. The "step height" between density phases in the floors of such loci was always greater than any polishing-induced topography between the same phases in adjacent, unresorbed areas (Figs. 10–12). When substrata in which the collagen fringe lining resorption loci was still intact were subjected to similar analysis, a similar step in the level of the fringe could sometimes be demonstrated, i.e. the collagen fringe appeared to be lower on the less densely mineralised side of a reversal line. However, this result was not found consistently and was sometimes largely attributable to polishing.

"Non-excavating" osteoclastic activity

There was evidence from the examination of the polished femoral slices that some osteoclastic activity had occurred without the production of frank resorption loci. This was evident as patches or tracks on the substrata, which appeared darker in the BSE image than the surrounding bone (Fig. 13). Patches were often associated with resorption complexes and tracks sometimes linked two adjacent complexes. SE mode images of these areas revealed a range of appearances. Sometimes they were smooth and showed little evidence of surface disturbance. Other areas presented a fine fibrillar appearance, which was absent when the specimen was made anorganic, thus demonstrating that superficial demineralisation had occurred in these areas. The examination of specimens fixed with the cells in situ showed that osteoclasts were frequently no longer associated with these "non-excavated" areas, indicating that cells which had been active at these sites had either moved on in vitro, or were poorly adherent and were dislodged during processing.

Other evidence of superficial, or early, osteoclastic activity were dark rings that were occasionally seen in BSE images of specimens from which the cells had been removed (Fig. 14). Corresponding SE images again demonstrated a variety of appearances, ranging from an apparently intact substrate surface to a ring of resorption surrounding a less affected (or apparently unaffected) central area (Fig. 15). These rings, which were not always complete, occurred both in isolation, when they were round, and in association with previously resorbed areas, when they outlined new kidney-shaped resorption loci (Fig. 14). They were found on osteoid (Fig. 6) as well as on mineralised bone matrix.

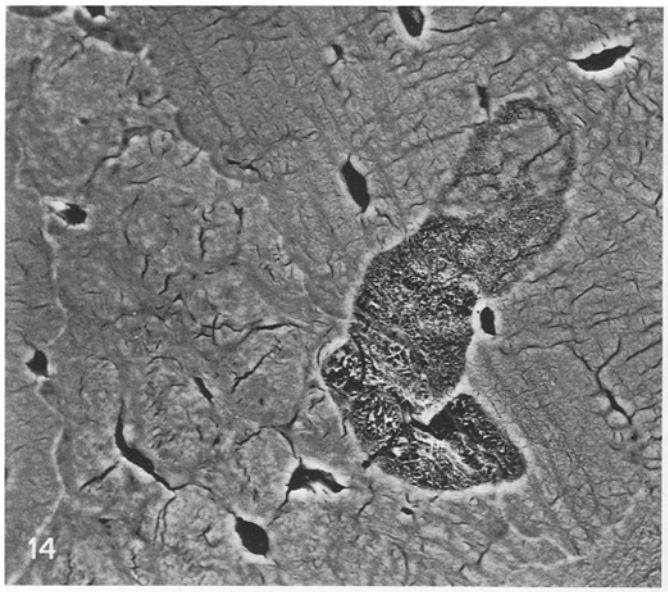
The annular zone

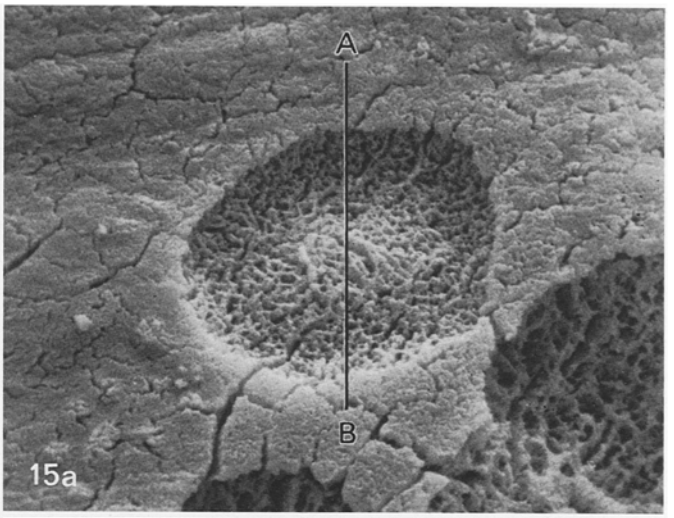
An annular zone around defined resorption loci (Jones et al. 1985) was observed both at the junction between adjacent loci within a complex and on the polished surface at the periphery of complexes. In neither situation was it a (noticeable) feature of all loci and, where it occurred, the zone was not always a complete ring around a locus (or complex). On polished bone surfaces the annular zone was evident in BSE images as a dark band (Fig. 13). Sometimes it correlated with a "gutter" around resorption loci, which could be seen in SE mode images, particularly in anorganic preparations. In other instances, no correlating feature was seen in SE images, the surface around resorption loci appearing undisturbed.

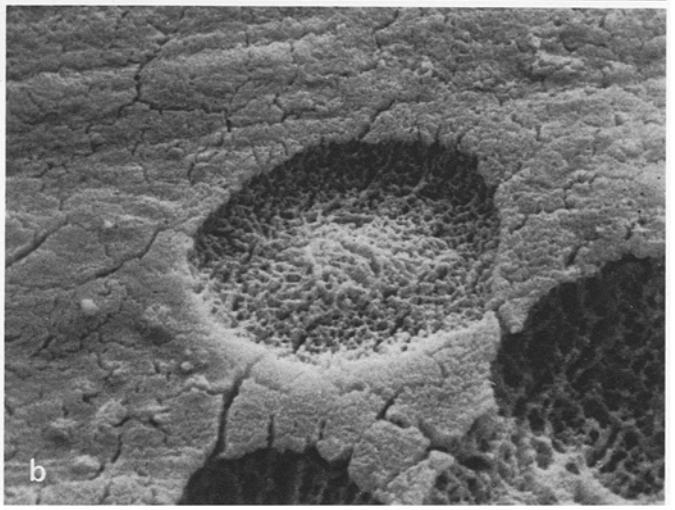
Fig. 10a, b. Stereopair showing a resorption complex formed during a 120-h culture of chick osteoclasts on a polished slice of human femur. Several loci span a reversal line separating less densely mineralised bone (*top*) from more densely mineralised bone. *AB* and *CD* indicate the positions of profiles across the reversal line that are shown in Figs. 11 and 12. Anorganic preparation. Rotate 90° anti-clockwise to view. Fieldwidth 75 μm

Figs. 11, 12. Profiles along lines AB and CD indicated on Fig. 10. *AB* shows the topography which is introduced by polishing; *A* less densely mineralised bone. *CD* shows the topography present at the reversal line after resorption. This step is four times greater than that introduced by polishing and shows that demineralisation has extended further into the less densely mineralised bone during a single resorptive episode. The *arrows* indicate the position of the reversal line

Fig. 13. A 10 kV BSE image of resorption on a polished femoral substratum incubated with osteoclasts for 48 h. *Dark bands* (annular zones) are present around some resorption loci. Also, superficially demineralised patches can be seen (*arrows*). Fieldwidth 170 μm







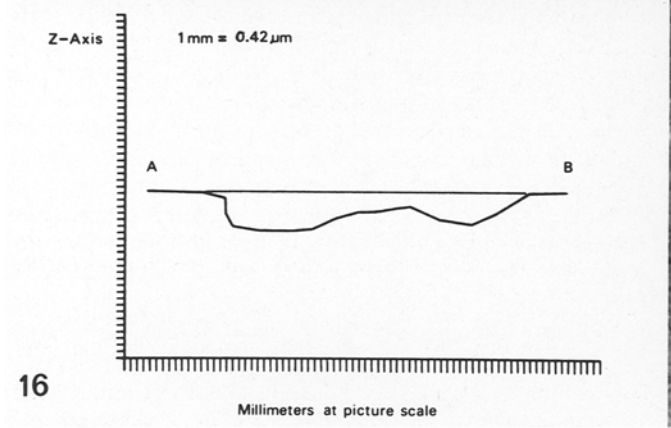


Fig. 14. A 20 kV BSE image, showing a ring of demineralisation, which probably represents the site to which the osteoclast forming this complex had most recently moved and begun resorbing; 120-h culture of chick osteoclasts on polished femoral slice. Fieldwidth 155 µm

Fig. 15a, b. Stereopair showing a resorption locus formed during a 96-h culture of chick osteoclasts on a human rib slice. The gradient of activity from periphery to centre (see Discussion), which may be established at the commencement of resorption, has been maintained despite substantial demineralisation. Anorganic preparation. Rotate 90° anti-clockwise to view. Fieldwidth 45 µm

Fig. 16. Profile across the resorption locus shown in Fig. 15. The *horizontal line* represents the original substratum surface

Discussion

These results show that chick osteoclasts, activated in vivo and then transferred to a situation of unrestricted substratum access, will resorb all matrix phases in sectioned human bone irrespective of whether they are mineralised or the degree of mineralisation. Their activity does not appear to be influenced by encountering changes in mineral density of the substratum, since they will continue resorbing across reversal lines whether the bone on to which they are moving has a higher or lower mineral content than that which they have already resorbed. A trend was observed towards increased area of individual resorption complexes with increased mineral density of the bone in which they occurred. Although this was not statistically significant, the result does indicate that a more detailed examination should be made of the variation in the plan extent and volume of individual resorption loci, and of the number of scoops per complex, in bone with different mineral contents.

Previous observations have suggested that the cutting cone advances without regard for the tissue architecture of the existing bone (McLean and Rowland 1963; Johnson 1966) and that osteoclasts are not influenced by the age and mineral density of the bone matrix they are resorbing (Young 1963). However, Tappen (1977) has described cutting cones which appeared to avoid the territory of newly deposited osteons (see, for example, his Fig. 2). The present results do not support the view that osteoclasts at a cutting cone would select older bone fractions to resorb simply because they are more mineralised. The results agree with those of studies of the resorptive activity which occurs at modelling surfaces during bone growth (Jones et al. 1985; S.A. Reid, unpublished work). Here osteons with different mineral densities are removed at grossly similar rates. These results fail to support any speculation that when a certain, dense level of mineralisation is achieved, bone becomes non-resorbable and therefore leaves the remodelling pool.

The failure of an osteoclast to cross a reversal line may

sometimes have resulted from its interaction with actual topography, but is more likely to have been due to contacts with other topography-aligned cells: in the absence of these, the direction of osteoclast travel during resorption is not influenced by grooves on a substratum (Jones et al. 1985). In the present study, alignment of resorption with substratum surface grooves only occurred in areas also populated by aligned osteoblasts and fibroblasts.

The profiles of the bases of resorption loci spanning reversal lines (Fig. 12) demonstrated that the rate of demineralisation of bone matrix during resorption (in terms of depth per unit time) is inversely related to the mineral density of the phase being resorbed. This agrees with earlier observations of dentine resorption, which showed that peritubular dentine stands proud of surrounding, less densely mineralised tissue at the bases of resorption loci formed in vivo (Boyde and Lester 1967) and in vitro (Jones et al. 1984).

The finding that cultured chick osteoclasts resorbed cut surfaces of unmineralised human bone matrix was not unexpected, since it has been demonstrated that unmineralised predentine and demineralised dentine substrata are resorbed by rabbit osteoclasts in vitro (Jones et al. 1984) and that osteoid may be resorbed in vivo (Boyde and Hobdell 1969; Jones 1973). Also, it has been reported that demineralised bone implants are resorbed in vivo by cells morphologically similar to osteoclasts (Glowacki et al. 1981; Irving and Handelmann 1963; Irving and Heeley 1970; Yakagi et al. 1979). However, this does not address the problem as to whether osteoclasts will invade an intact osteoid surface in vivo.

The observation of superficial demineralisation peripheral to the edge of some resorption loci (Fig. 13) was predictable, although it has recently been reported that bone at this location remains completely undisturbed during resorption (Chambers 1985). This phenomenon was, in fact, first described in an SEM study of in vivo resorption when shallow gutters were noted bounding a number of resorption loci in mouse and rabbit bone (Jones 1973). Similar guttering has been found associated with resorption of bone and dentine occurring in vivo in a number of other mammalian species and in bony fish (S.J. Jones, unpublished work); dark rings of demineralisation, evident in BSE images, have been described around resorption loci formed in vitro on dentine slices (Jones et al. 1985). The cause of the annular zone is not known. It may represent demineralisation deep to, or beyond, a static osteoclast clear zone or be the result of a small shift in the position of this zone during a resorptive episode (Jones et al. 1985). It does not appear to be associated with a particular resorption locus morphology, being found around large, small, deep and shallow embayed areas.

The wider hoops of demineralisation, or shallow excavation, which were recorded on some specimens (Fig. 14), were similar to those found by Jones et al. (1985) on carbonate-containing mineral substrata resorbed in vitro. These authors have suggested that the rings show the inner limit of the clear zone of the osteoclast and, therefore, indicate that the area over which a locus extends is usually set at the beginning of a resorptive event. The presence of such rings on osteoid, as well as mineralised bone matrix, in the present study demonstrates that secretion of matrix degradative enzymes, as well as protons, may be initiated from the periphery of the ruffled border. Also, it is evident that this hoop morphology (with the periphery more deeply resorbed than the centre) may be maintained until resorption loci are quite well established (Figs. 15, 16), indicating that the lag in onset of activity across the ruffled border can be substantial.

The patches and tracks of superficial osteoclasis demonstrate that significantly more osteoclastic activity occurs at the surface of substrata than is indicated by the amount of frank resorption seen. Such superficial, or transitory, activity may be common to all osteoclasts, although nothing is yet known of the behaviour of normal osteoclasts from adult, or aged, animals in the culture system used here. Its recent recognition may be due to the combined use of BSE imaging and suitable (i.e. smooth or polished) substrata for in vitro studies. Clearly, the use of SE imaging to examine rough substrata is not sufficient to permit a conclusion of osteoclast inactivity, since demineralisation in the absence of cavitation may go unrecorded.

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