MOTION PICTURE AND ELECTRON MICROSCOPE STUDIES ON THE EMBRYONIC AVIAN OSTEOCLAST

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ABSTRACT

Time-lapse motion picture studies were carried out on isolated fowl embryo osteoclasts in vitro; the cells have an extremely active ruffled border, and show vigorous pinocytotic activity. Electron microscope studies on osmium-fixed cells showed that the pinocytotic vacuoles contained bone salt crystals (as well as material which could not be identified on morphological grounds), and that the folds of the ruffled border enclosed crystals and collagen fibrils. Changes were seen in the matrix beneath the ruffled border. Initially, the collagen fibres became separated from each other and at the same time bone salt crystals became detached from them. Later, as crystals and ground substance disappeared, the outline and cross-striation of the collagen became distinct. The implications of these findings are discussed with respect to the mechanism of bone erosion.

Until recently it was open to question whether or not osteoclasts take any active part in bone absorption (14, 16). Now, however, this seems practically certain. Gaillard (5-7) and Goldhaber (8-11) have both filmed bone absorption in vitro; osteoclasts can be seen moving around on the bone which appears to melt away beneath them. Arnold and Jee (1) administered plutonium to dogs and prepared autoradiographs of bone sections. Osteoclasts became labelled, having taken up a dose from subjacent bone. Electron microscope evidence, too, indicates that the cells attack bone. The first work reported was that of Scott and Pease (29); working with kitten material, they identified crystals of bone salt within the "ruffled border" and thought that the matrix showed morphological changes. Cameron and Robinson (3) identified bone salt crystals in the cytoplasm of human osteoclasts. More recently, Gonzales and Karnovsky (12) reported their results with the rat. These, in some respects, confirmed the findings of Scott and Pease concerning the morphological aspects of bone absorption.

Whilst this new evidence leaves little room for doubting that osteoclasts play an active role in bone absorption, their precise mode of action remains in many respects as obscure as ever. The observations described below, which collate work on the living cell with electron microscopy of the fixed cell, carry knowledge a little further. The results with living cells suggest that movement of the ruffled border and pinocytosis are important in osteoclast function, whilst the electron microscope studies, the results of which differ in some respects from previous reports, indicate that bone absorption reflects the stages of bone deposition but in reverse order.

MATERIALS AND METHODS

A. Time-Lapse Motion Picture Studies

The basic technique was the same as that in earlier experiments (13, 15, 17). Small pieces of embryonic frontal bone were explanted in plasma clots. Osteoclasts migrate outwards from the bone into the clot; cinematographic records of the be-
haviour of such isolated cells were made with phase-contrast illumination.

B. Electron Microscopy

Small pieces of the frontal process of the 14-day fowl embryo frontal bone were fixed in ice-cold 2 per cent buffered osmium tetroxide (Palade, 24) for 3 hours, rinsed in water, and dehydrated in ascending grades of alcohol which had been alkalized with magnesium carbonate to reduce the risk of decalcification, and embedded in s-butyl methaerylate. Sample sections were cut with a diamond knife on a Porter-Blum microtome at approximately 0.5 μ thickness, and mounted on slides. These were stained with alcohol safranin, rinsed in water, dried, mounted in liquid paraffin, and searched with the phase-contrast microscope for osteoclasts. If the latter were present, thin sections were then prepared consecutively for electron microscopy; if not, the tissue block was sampled again at progressive depths. Attempts to study the same osteoclast in serial thin sections met with limited success; it was not always easy to obtain ribbons of undamaged sections of the bone, and, in any case, the cell to be studied often came to lie behind the intersections of the supporting grid bars. Electron micrographs were prepared in a Siemens Elmiskop I.

RESULTS

A. Motion Picture Studies

The isolated cells sometimes adhered to the coverslip and spread out wholly or partially upon it. Cytological details stood out clearly in these instances (Fig. 4); the long mitochondria, the nuclei with one or two nucleoli, cytoplasmic vacuoles and ruffled border could all be followed as the cells moved. Details of the structure and mode of progression of osteoclasts *in vitro* have been published previously (13, 15).

Three aspects of the isolated cells *in vitro* require description. First, they possessed an extremely

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**Explanation of Abbreviations**

- ab, altered bone
- nb, normal bone
- d, damage
- pv, pinocytotic vacuole
- j, junctional zone
- rb, ruffled border

1 to 5 are enlargements from the 16 mm film records; the remainder are electron micrographs.

**FIGURE 1**

A part of a living osteoclast *in vitro*. Towards the right the cytoplasm has spread out as a thin layer. Pinocytotic vacuoles are seen in process of formation along that edge, whilst older vacuoles are located nearer the centre where out-of-focus nuclei, and mitochondria, are seen. The cytoplasm extends upwards out of the field of view. × 1,000.

**FIGURES 2 AND 3**

These are of the same cell, time interval approximately 30 seconds. One nucleus (with nucleolus) and surrounding cytoplasm are visible. Note thin, folded veils of ruffled border and pinocytotic vacuoles in Fig. 2, and their changes in Fig. 3. × 1,000.

**FIGURE 4**

Portion of an osteoclast *in vitro*. Six nuclei are visible in the centre, surrounded by mitochondria. Folds of the ruffled border can be seen above and below on the left; a pinocytotic vacuole is present at upper right, and others near nuclei. × 1,000.

**FIGURE 5**

An osteoclast, which contained 8 nuclei, showing the folds and veil-like processes of the ruffled border, especially along the bottom edge. × 400.
active and extensive undulating membrane. This thin veil-like zone of peripheral cytoplasm was in continuous energetic movement. Tongue-like processes of cytoplasm of varying size were thrown out. They seemed to fold and unfold and to sweep and explore their vicinity before being withdrawn and replaced. Still pictures give a very incomplete idea of this restless activity at the cell surface (Figs. 1 to 3, 5).

Second, the cells exhibited vigorous pinocytotic activity (Figs. 1 to 4). Vacuoles of varying size were seen to be formed in the classical manner by the undulating membrane and to travel towards the interior. They persisted for varying periods before fusing with others or disappearing abruptly, presumably by discharging their contents to the cell surface. It is interesting that when powdered "deproteinized" bone (18) was added to the culture medium, particles appeared within the cells inside pinocytotic-like vacuoles.

Third, from time to time, as thin cytoplasmic processes formed and withdrew, occasional

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**FIGURE 6**
Portion of an osteoclast above, altered bone below, and junctional zone separating the two. At top left, part of a pinocytotic vacuole containing bone salt crystals. Free bone salt crystals in the junctional zone, and between the collagen fibers which have become separated from one another. Separation of the fibers seems greatest near the junctional zone. X 21,000.

**FIGURE 7**
Higher-power view of portion of another cell, illustrated also in Fig. 11. Note channels in ruffled border; these contain bone salt crystals which can also be seen in the junctional zone below. Towards bottom is altered bone. There has been damage on sectioning at d. X 28,000.
glimpses were caught of much finer cytoplasmic vacuoles, the size of which was at about the limit of visibility. These probably corresponded to similar structures seen in electron micrographs of fixed cells.

B. Electron Microscopy

For the sake of brevity, description will be confined to the cytoplasm adjoining the bone, the subjacent bone itself, and the junctional zone between the two. The authors hope to publish later a full account of the ultrastructure of the cytoplasm and its inclusions.

1. Altered Bone: Structural changes in the bone were confined to the matrix beneath the ruffled border of the cells. With some cells, comparison of serial sections indicated that the affected zone was saucer-shaped. Peripheral regions of this zone were less affected than the central region, which suggests that the change began at a particular point and later spread to the surrounding matrix. With other cells, a much deeper pit had formed, and the bone showed more advanced changes as described below.

The earliest change detected consisted of the separation of collagen fibres so that spaces appeared between them, and the occurrence of detached bone salt crystals. The latter were visible in the spaces between fibres, and at the surface of the affected area (Figs. 6, 7, 13).

In serial sections of the same cell, alterations in the bone matrix extended more deeply as the section plane progressed and, also, became more pronounced. Loss of bone salt crystals from the more superficially placed fibres had progressed and many free crystals lay in the interfibre spaces and between bone surface and cytoplasm. In addition, the outlines of the fibres had begun to become clearly distinguishable; and, eventually,

**Figure 8**

Low-power view of ruffled border, junctional zone, and altered bone. Naked collagen fibrils can be identified in centre of junctional zone, where advanced changes are present. × 12,000.

2. Junctional Zone: Spaces of varying shape and
size separated the surface of the altered bone from the ruffled border of the osteoclast. Since this junctional zone had an extremely low electron density, it probably consisted of "tissue-" or "extracellular-fluid" in life. It contained varying numbers of loose bone salt crystals and protruding into it were also, of course, the ends of the collagen fibrils (Figs. 6, 8, 11, 13).

3. CYTOPLASM: Bordering the altered bone substance was the ruffled border of the osteoclast (29). This consisted of innumerable cytoplasmic processes and folds arranged in a pattern of extreme complexity (Figs. 6, 8, 11, 13); channels led off towards the cell interior here and there (Figs. 7, 11, 13), reflecting pinocytotic activity. In general, the appearance of the ruffled border agreed well with the descriptions and illustrations given by Scott and Pease (29), but in the present material its outer contour seldom followed that of the subjacent bone so closely. Usually it was separated, as the illustrations show, by quite large spaces, as if blebs of fluid had been present. On the other hand,

FIGURE 9
Higher-power view of portion of preceding. Ruffled border above. Note cross-banded collagen fibrils cut longitudinally; others are cut obliquely. Free crystals are present. × 32,000.

FIGURE 10
Higher-power view of another portion of Fig. 8. Ruffled border above, altered bone below. At bottom right the bone is almost normal, but upwards and to the left the fibres have become separated, then stripped of crystals. In places, transections of naked collagen fibrils are partly surrounded by membrane folds (arrows). Free crystals between fibrils and ruffled border. × 32,000.

As described by Scott and Pease (29) and by Gonzales and Karnovsky (12), bone salt crystals were identified in channels and folds of the border (Figs. 7, 12). In addition, in the present material, collagen fibrils were seen to be invested by folds and processes of the border (Figs. 8, 10, 13, 15). However, collagen was never seen inside the cell.
and must presumably have been digested quickly within or outside the ruffled border.

As reported for the kitten (29), the cytoplasm of the embryonic fowl osteoclast was found to be extremely vacuolated, more so than in the rat, judging from the illustrations published by Gonzales and Karnovsky (12) for the rat. The functional significance of the small vacuoles remains obscure at present but the larger were pinocytic (Figs. 1, 14, 6). In both light (Figs. 1, 3) and electron (Fig. 14) micrographs their diameter often measured roughly 2 micra. They contained inclusions of varying appearance. Frequently, bone salt crystals could be recognized from their appearance; in some instances, the subjective impression was gained that the crystals had become shorter within the vacuoles, as if in process of solution. Often the nature of the vacuolar contents could not be diagnosed on morphological grounds (Fig. 14). Collagen was never identified.

It should be added that changes in the bone matrix were always confined to a restricted zone defined roughly by the ruffled border; elsewhere, it appeared normal. This confirms the findings of Scott and Pease (29) and of Gonzales and Karnovsky (12).

**DISCUSSION**

Dealing first with the motion pictures, the fact the osteoclasts isolated in vitro show vigorous movement of a ruffled border and active pinocytosis does not, of course, prove that similar events take place in the intact animal. However, it is a matter of everyday experience that vacuoles are seen with the light microscope in the border cytoplasm of osteoclasts in fixed and stained tissue sections. They have been discussed in recent years by Kroon (20). In the light of the motion picture findings described above, it seems safe to conclude that they are pinocytic in nature. From the film records, too, it seems very likely that the classical "brush" or "striated" border of the osteoclast seen in fixed tissue sections is simply a view of the folds of the undulating membrane complicated by technical artefacts and section plane difficulties. It seems justifiable to conclude that much energy is expended by the cell in active cytoplasmic sweepings of the bone surface and that exposed collagen fibres when seized by cytoplasmic processes are shifted around and possibly even vigorously shaken.

The electron micrographs indicate that bone salt crystals were gathered up by ruffled border folds, and conveyed to the interior of the osteoclast in pinocytic vacuoles. It now seems probable that the vacuoles containing crystals reported in human cells by Cameron and Robinson (3) were of this nature. Within the pinocytic vacuole, the crystals are no doubt reduced to a soluble form, and, when it discharges, the mineral is presumably shed into the extracellular fluid and finds its way into the bloodstream. It is possible, although there is no direct evidence, that the products of extracellular digestion of collagen and ground substance might be gathered up in the same general way. However, it is also possible that instead of being removed in solution, digestion products might "lock-on" to specific receptive sites on the cell membrane and be actively transported inside the cell by pinocytic infoldings of these membrane sites; dye and protein molecules have been shown to behave in this way with amoebae (2, 27, 28).

To speculate further, it is possible that intracellular enzymes might, as suggested by Rose (26), be liberated into the pinocytic vacuoles; these enzymes might be active within the vacuole, or be shed to the surface when the latter discharges and be carried to the bone surface by movement of the ruffled border to act upon the matrix.

As mentioned above, the significance of the countless small cytoplasmic vacuoles is at present quite unknown. All that can be said is that the refractility of the cytoplasm of the living cell examined in vitro in transmitted light (13) is probably due to their presence.

The separation of the collagen fibres, which seemed to be the earliest demonstrable change in the matrix, requires some discussion. The basic question which is bound to arise is whether this separation was real or an artefact generated when the sections were cut. Its constant occurrence beneath the ruffled border and its absence elsewhere suggest, at the very least, that there must have been some underlying change in the physical state of the bone. Although the configuration of the fibre separation did not correspond to the axis of the knife, the extent to which mechanical factors in sectioning had distorted the morphology of altered matrix must unfortunately remain a matter of opinion and of work by others. Scott and Pease (29) also report rather similar changes in this area, although in their illustration 15a some mechanical shredding of the bone certainly seems a distinct possibility.
On general grounds, the stages in the removal of bone might be expected to follow those of bone formation in reverse order. In bone deposition, collagen fibrils appear first; initially separated, they soon mat together, through the appearance of ground substance, to form the preosseous matrix; this finally calcifies (4, 29, 25). In the present work, absorption sequences seemed to have mirrored these stages, more or less in reverse order. First, there was loss of crystals together with separation of collagen fibres; next, there was the "unmasking" of collagen striation; and finally, the presumptive solution of the protein. Since neither Scott and Pease (29) nor Gonzales and Karnovsky (12) observed collagen free of crystals they naturally maintained that the fibres disappear first. This interesting discrepancy could perhaps be explained in two ways.

First, it might be argued that the previous workers were dealing with the initial stages only of bone erosion. Hence, their illustrations show detached bone salt crystals but not exposed collagen because either the cell had only just begun to operate, or the section plane did not pass through the site of maximum activity. In the present studies, naked collagen was seen beneath the ruffled border only when the section plane had progressed to a certain depth. Second, it might be inferred that a genuine species difference has been brought to light, as between the kitten and the rat on the one hand, and the embryonic fowl osteoclast on the other. Although possible, this does not seem very likely especially since recent work by Cameron (30) with the guinea pig supports the present findings. Meantime, it should be pointed out that there is a basic difficulty to keep in mind whilst interpreting electron micrographs of osteoclasts or any other cell with actively moving cytoplasmic processes; when tissue is chilled on transfer to ice-cold fixative, there is always the possibility that cell processes will quickly retract to produce a final picture quite unlike that of the living cell. This requires critical evaluation.

The nature of the changes which loosen the crystals and lead to separation of the fibres remains to be identified. Kolliker (21) himself thought that the likeliest explanation for the demineralization of bone was the production of an acid by the osteoclast, and this idea has been perpetuated in various forms ever since. Recently, of course, citric acid has been mentioned (23). However, free bone salt crystals have now been seen in three different laboratories and in three different species. It is difficult to reconcile their occurrence with the concept of acid decalcification of the surface of the matrix.

It seems likely, of course, that the altered bone must become increasingly hydrated as the changes

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**Figure 11**
Normal bone below (some damage and folding due to sectioning). Altered bone beneath ruffled border forms a saucer-like depression (a portion only is shown) wherein separation of collagen fibres and shedding of crystals can be distinguished. Note almost complete absence of changes in bone below osteoclast cytoplasm lacking ruffled border. × 14,000.

**Figure 12**
High-power view of ruffled border showing bone salt crystals gathered up in membrane fold. Free crystals also in junctional zone. Altered bone below. × 52,000.

**Figure 13**
Ruffled border above, bone below. To left, the collagen fibres below the border have become separated. Towards the centre of the picture the surface of the bone shows more advanced changes and bare collagen fibres (cut both transversely and longitudinally) can be identified. Meantime, more deeply, early changes have commenced. In several instances the same fibre (f) can be traced as it passes through zones of early change to a point where its cross-banding is distinctly seen.
Note also collagen fibre (recognized from its cross-banding) gathered up in membrane folds. × 24,000.
FIGURE 14
Active portion of an osteoclast showing typical large pinocytotic vacuoles containing crystals. Other (smaller) vacuoles contain material of unknown nature (arrow). Mitochondria and part of a nucleus are present; collagen fibrils and altered bone visible near bottom. X 9,000.

FIGURE 15
Profiles of transected collagen fibrils of various sizes (arrows) invested in places by ruffled border fold. X 32000.

...proceed in depth. In this connection the view expressed above that collagen is dissolved only after the crystals have been shed seems to gain support from a suggestion recently put forward by Neuman et al. (22): they maintain that the limited diffusion possible in calcified bone would prevent the inward passage of complex protein molecules such as proteolytic enzymes. When defatted, powdered bone was treated with a variety of proteolytic enzymes, no loss of nitrogen occurred. However, if the powder was first demineralized, the enzymes were active in proteolysis of the matrix.

It has been shown by Jowsey et al. (19) that radioactive yttrium deposits preferentially at sites of bone absorption. The biophysical and biochemical aspects of the way in which yttrium attaches to bone have been investigated by Neuman et al. (22). They report that apatite crystals, and therefore, bone mineral, have a "fantastic affinity" for yttrium. It may very well be that the demonstration of free bone salt crystals beneath osteoclasts by electron microscopy provides the explanation for the localization pattern of yttrium.

Finally, it should be stressed that although the osteoclast is now firmly established as being actively involved in the removal of altered bone, we are still ignorant as to whether it alters the bone itself or is simply attracted to altered loci.

Received for publication, July 2, 1961.

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