THE PROBLEM OF DEMINERALISATION IN THIN SECTIONS OF FULLY CALCIFIED BONE

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ABSTRACT

Thin sections of embryonic avian bone decalcify during preparation for electron microscopy, creating a false impression of mineral distribution. The results of the experiments reported herein show that viscous embedding materials do not penetrate compact formed bone, and so, in thin sections, the calcium apatite crystals may be leeched out by water, both in the collecting trough and in aqueous solutions of stains used to enhance tissue electron opacity. To prevent decalcification, a simple technique is described in which the aqueous fluids that come in contact with thin sections are saturated with respect to calcium and phosphate ions, thereby preventing solution of the bone mineral. The theoretical basis of this technique is briefly discussed.

INTRODUCTION

No doubt because of the technical difficulty in preparing thin sections of calcified tissue for examination by electron microscopy, relatively few reports have been published dealing with bone and related materials. In these reports there seems to be little discussion of the possibility of artefacts caused by demineralisation of the calcified matrix during routine preparation of the specimen. Dudley and Spiro (1961) stated that stained sections, because of the action of staining fluids of various pH values and ionic concentrations showed some degree of demineralisation when compared with unstained sections from the same block. Durning (1958) collected his specimens onto what he terms "partly saturated tricalcium phosphate solution" to reduce the solution of inorganic crystals, but does not elaborate on this statement.

The present report concerns current work on this problem and includes the description of a technique whereby demineralisation may be prevented.

METHODS AND OBSERVATIONS

Small pieces of the frontal process of the frontal bone of 14-day fowl embryo were fixed in ice-cold 2 per cent buffered osmium tetroxide (Palade, 1952) for 3 hours, rinsed in distilled water, and dehydrated in ascending grades of alcohol. The specimens were then transferred *via* toluene (1½ hours) and toluene/Araldite 50/50 mixture (1½ hours) into Araldite, three changes of resin mix for 3 hours each at 35°C being used to effect infiltration before final embedding in gelatin capsules.

Sample sections 0.5 μ thick were stained with 50 per cent alcoholic aqueous safranine, rinsed in water, mounted in liquid paraffin, and examined by phase contrast microscopy for suitable regions. The block face was then reduced in size to give sections about 300 by 150 μ for electron microscopy.

Sections were cut by a diamond knife, initially onto distilled water, in a Huxley pattern Cambridge ultramicrotome and mounted serially on grids. A Siemens Elmiskop I was used to examine the specimens.

The effect of time of exposure to water upon the appearance and distribution of bone salt crystals was studied as follows. With zero time being taken as the instant before the first section appeared from the knife, sufficient serial sections were cut fully to load one grid (about 15 sections). Every effort was made to ensure even thickness in the sections; by interference fringes, this was about 600 A (silver-grey

colour (Peachey, 1958)). The microtome was set to cut at 400 A. The sections were spread with chloroform vapour (time T_s) and collected on a grid (time T_c). The time at which the last section of a ribbon was cut was also noted (T_l). All intervals were measured from zero time.

The effect of section thickness was examined by varying the microtome thickness setting above 400 A within narrow limits, but, in the main, results were gained from sections cut at 400 A, the minimum setting at which consistently good sections were obtained. It was found that mineral in thicker sections (cut at 600 to 700 A) took longer to dissolve than that in sections cut at 400 A, as might be expected.

On the Cambridge ultramicrotome, the rate of fall of specimen past the knife can be varied by altering the viscosity of an oil contained in a dashpot. To prevent folds occurring in sections of Araldite cut by a diamond knife, the rate of fall of a specimen past the knife must be very slow—the oil used in these experiments was of such viscosity that one complete cycle of microtome operation took about 12 seconds. Usually, about 15 sections could be placed on a grid and, including the spreading and grid loading operations, the first section of the ribbon cut was floating for a minimum of 3½ minutes (Table I, No. 6). Under normal working conditions in most laboratories, it is usual for two or possibly three grid loads of sections to be cut before collection commences, and so sections may float for 5 minutes or more before recovery. Again, it is well known that epoxy resin-embedded tissue often, in thin sections, requires heavy metal staining to increase contrast to acceptable levels. Such staining techniques may require from 10 minutes to 2 hours for full effect, and thus the sections are in contact with aqueous solutions for sufficient time, as will be shown, to cause complete demineralisation of the bone.

TABLE I

Experiment No.	Thickness testing	T_l		T_s		T_c		Results
	A	*min.*	*sec.*	*min.*	*sec.*	*min.*	*sec.*	
1	400	2	50	3	50	30	00	Decalcified
2	400	3	10	10	00	14	15	Decalcified
3	400	2	35	6	00	9	00	Decalcified
4	400	2	25	2	45	5	10	Undecalcified
5	500	2	10	2	30	4	55	Undecalcified
6	500	1	30	1	40	3	30	Undecalcified
7	400	2	30	2	50	32	00	Undecalcified
8	400	2	30	30	00	32	00	Undecalcified

T_l, T_s, and T_c refer to times measured from zero time at which:

T_l, the last section of a ribbon was cut.

T_s, the ribbon was spread with chloroform.

T_c, the ribbon was collected and dried.

The term *Decalcified* indicates that bone trabeculae were devoid of crystals of bone mineral, except near active bone-modelling cells as explained in the text, and the random weave of collagen fibrils of the bone matrix was clearly visible.

In experiments 7 and 8, sections were cut onto water saturated with calcium phosphate. In the other experiments, the fluid in the trough was distilled water. At least 40 sections (3 grid loads) were examined in each experiment.

Table I indicates the time intervals of completed tests, in which sections floated for periods of up to 30 minutes. The time T_s was varied to check whether sections spread by chloroform as soon as possible after cutting were more rapidly demineralised than sections left floating in the compressed state caused during sectioning. However, compared with the length of time for full demineralisation (6½ to 8 minutes in sections about 600 A thick), the increase in time for full demineralisation with a high T_s value was barely noticeable, being a maximum of ½ a minute over a number of determinations.

The first three experiments were carried out to determine, as accurately as possible, the shortest time required for full demineralisation (Figs. 1 and 2), whilst the next three, in which cutting was done at a quicker rate, were attempts to collect good sections (relatively free from knife marks and folds) as quickly as possible and hence to avoid demineralisation (Figs. 3 and 4). The last two were tests using a collecting fluid which consisted of a saturated solution of calcium phosphate in distilled water (Fig. 5). This solution was prepared by adding sufficient calcium phosphate (calcium orthophosphate pptd., British Drug Houses, Ltd.) to saturate a stock volume of distilled water (about ½ gm per 50 ml: the solubility of this chemical is quoted later). It was allowed to stand for about 12 hours with occasional shaking before use. When required in the collecting trough, a suitable volume was filtered twice through Whatman No. 5 filter paper to prevent section contamination by the solid. Saturated calcium phosphate solution was also employed in a modification of the lead acetate stain of Dalton and Zeigel (1960), as follows. Sufficient calcium phosphate was added to the lead acetate solution (about ½ gm per 50 ml) to cause saturation. The solution was allowed to stand overnight to ensure that conditions of saturation existed, and sections were treated in accordance with the method of Dalton and Zeigel, except that the fluid for staining was twice filtered (through Whatman No. 5 paper) before use. Sections stained by this method showed the expected gain in contrast but, in addition, there was no apparent loss

of bone matrix (Fig. 6, compare Dudley and Spiro, 1961).

All tests were carried out at temperatures in the range 19 to 21 °C in a relatively draught-free room.

Unstained collagen fibrils in sections of osmium tetroxide–fixed tissue show relatively little density in electron micrographs in comparison with the osmiophilic cellular materials. In these experiments, where mineral was removed from sections, it was found that, in order to avoid "dodging" during printing and yet show the collagen fibril weave, photographs had to be prepared with low contrast paper (Kodak grade 1). Although the contrast of cellular components suffered by this process, the need for such action itself clearly demonstrates the demineralisation of the sections (Figs. 1 and 2), since bone mineral is electron-opaque and appears dense black in prints (Figs. 3 to 6). Photographs could be prepared from negatives of mineralised sections, using hard grade paper (Kodak grades 3 and 4), which yielded good contrast in the cellular components.

RESULTS AND DISCUSSION

As a result of the tests carried out, demineralisation, as assessed by visual examination of plates from sections of relatively thick (30 μ or more) bone trabeculae, took place in 6½ to 8 minutes in sections about 600 A thick. So far, insufficient data have been collected to give a more accurate time. However, since several factors, such as variable section thickness, purity, pH, and temperature of the collecting trough water, may have an effect on the final result, a more accurate figure would be meaningless. The mere fact that demineralisation occurs in so short a time as about 6½ minutes in these relatively thin sections means that, to prevent alteration or loss of bone mineral, rapid collection of the sections is essential. It appears that the loss of mineral which presumably occurs in the first 5 minutes or so is insufficient to be detected in electron micrographs.

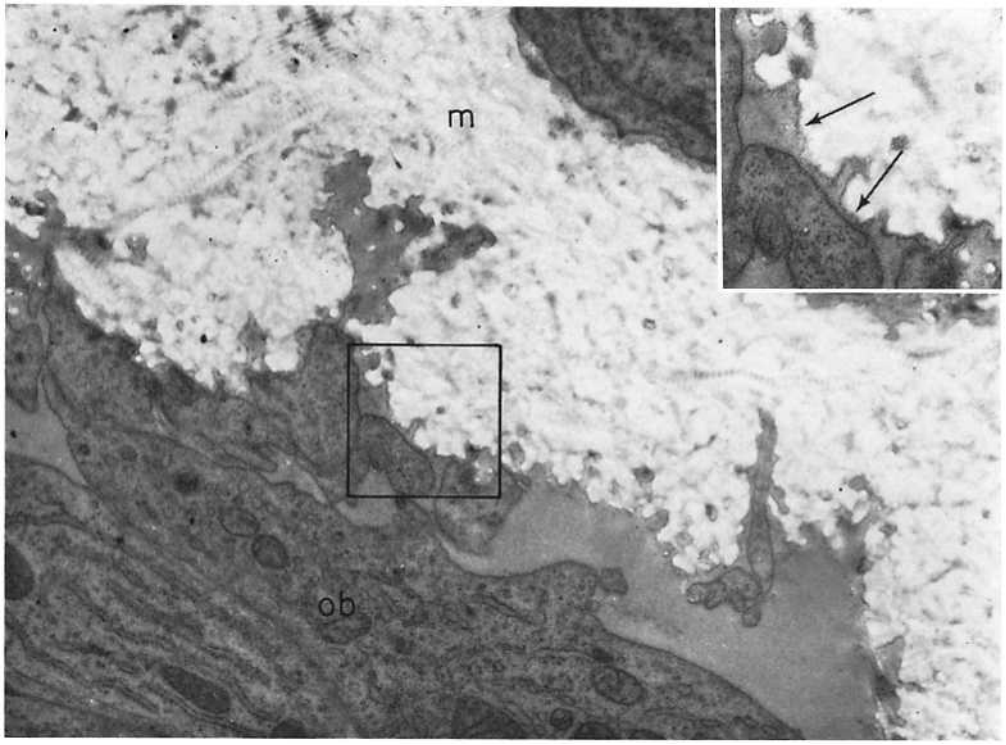

FIGURE 1 Part of an osteoblast (*ob*) and the adjacent bone trabeculum. Collagen fibrils in the matrix (*m*) show typical cross-striations indicating loss of bone crystals (compare Figs. 3 to 6). Section floated for about 12½ minutes on *aq. dest.* × 15,000, Kodak grade 1 paper.

Inset. Crystals retained by the embedding medium at the edge of the bone show as a dark line (arrows). × 30,000.

Whilst the fact that demineralisation takes place is shown by the change in appearance of bone trabeculae (*i.e.* disappearance of crystals, appearance of collagen fibrils) after treatment with distilled water, the actual removal of apatite crystals may occur in several ways, for example:

1. By dissolving in the water.
2. Physical stripping by a combination of the operations of sectioning and water contact.
3. Loosening by forces as in 2, and removal (shaking off) during subsequent dry procedures before exposure to the electron beam.

On the supposition that the bone crystals are removed by the solvent action of water only, the solubility of the crystals may be decreased by:

a. Introducing Ca^{++} ions into the collecting fluid, *e.g.*, by using a solution of a calcium salt, $CaCl_2$, for example.

b. Introducing $PO_4^{\equiv}$ ions, similarly, by means of a soluble phosphate, $(NH_4)_3PO_4$, for example.

c. Introducing both Ca^{++} and $PO_4^{\equiv}$ ions into the collecting fluid.

By assuming bone mineral to be composed *entirely* of calcium phosphate and using 10^{-26} as the value for the solubility product of bone mineral

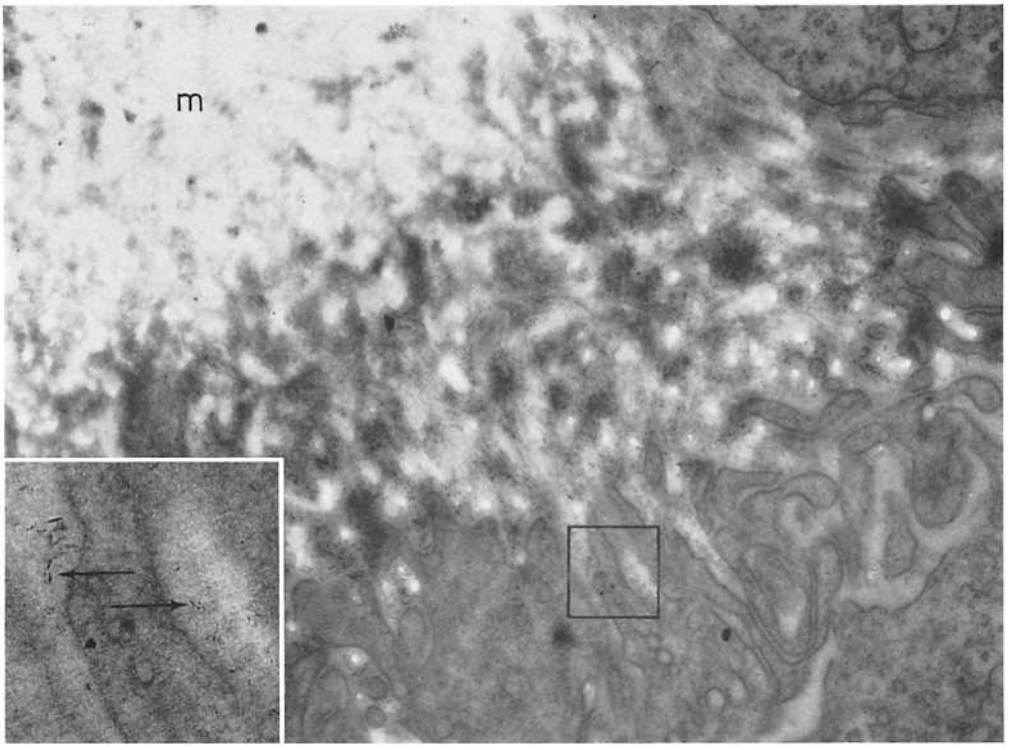

FIGURE 2 Active site of an osteoclast. The channels and vacuoles of the ruffled border contain crystals (see inset, arrows) and also collagen fibrils partially stripped of crystals. The bone matrix (*m*) near the cell's resorbing zone is demineralised and shows no sign of apatite crystals (compare Figs. 3 to 6). Section floated for about 7½ minutes on *aq. dest.* × 25,000, Kodak grade 1 paper.

Inset, × 80,000, Kodak grade 4 paper.

(see MacGregor and Nordin, cited in McLean and Urist, 1961), the effect of introducing such ions can be calculated.

In the following expressions, the square brackets are used to indicate concentrations in gram ions per litre. To obtain more accurate results, these concentrations should be corrected by the corresponding activity coefficients, but such corrections have been omitted since their use would not materially affect the discussion.

CASE A: Suppose the collecting trough water contains 0.01 gm ions of calcium per litre. Then,

from the solubility product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2 = 10^{-26}$, we have

$$[PO_4^{\equiv}]^2 = \frac{10^{-26}}{10^{-6}} = 10^{-20} \quad \text{whence} \quad [PO_4^{\equiv}] = 10^{-10}.$$

Initially, in distilled water saturated with calcium phosphate (bone mineral), $[PO_4^{\equiv}] = 5 \times 10^{-6}$. By converting these ionic concentrations to solubilities, it can be seen that a saturated solution of bone mineral is reduced in concentration from 7.67×10^{-4} gm/litre (in *aq. dest.*) to 1.55×10^{-8} gm/litre (in a solution containing 10^{-2} gm ions of calcium per litre), a factor of about 50,000 times.

CASE B: In a similar way, the addition of a phosphate, instead of a calcium salt, of the same concentration reduces the solubility of $Ca_3(PO_4)_2$ to about 4.8×10^{-6} gm/litre; in this case, the effect on the solubility is markedly less, *i.e.*, a factor of only about 160 times.

In both of the above examples, the salts introduced into the collecting trough water would, unless rinsed away in distilled water, appear as contaminant on the specimen when viewed in the electron microscope. By rinsing the grid the danger of dissolving the bone salt out of the section is reintroduced.

CASE C: By using calcium phosphate–saturated water in the collecting trough of the microtome knife, the solvent effect on the bone is eliminated. At the same time, the contaminant effect is virtually negligible; a saturated solution of calcium phosphate contains 7.67×10^{-4} gm/litre and, therefore, when one drop (0.05 gm) of this solution is dried on a carbon-coated grid only a very slight deposit can be resolved in the electron microscope. Since no crystalline structures have been found, it seems unlikely that this solution could lead to confusion by appearing as apatite crystals on the section. Furthermore, surplus fluid is normally drained from the loaded grid with filter paper to

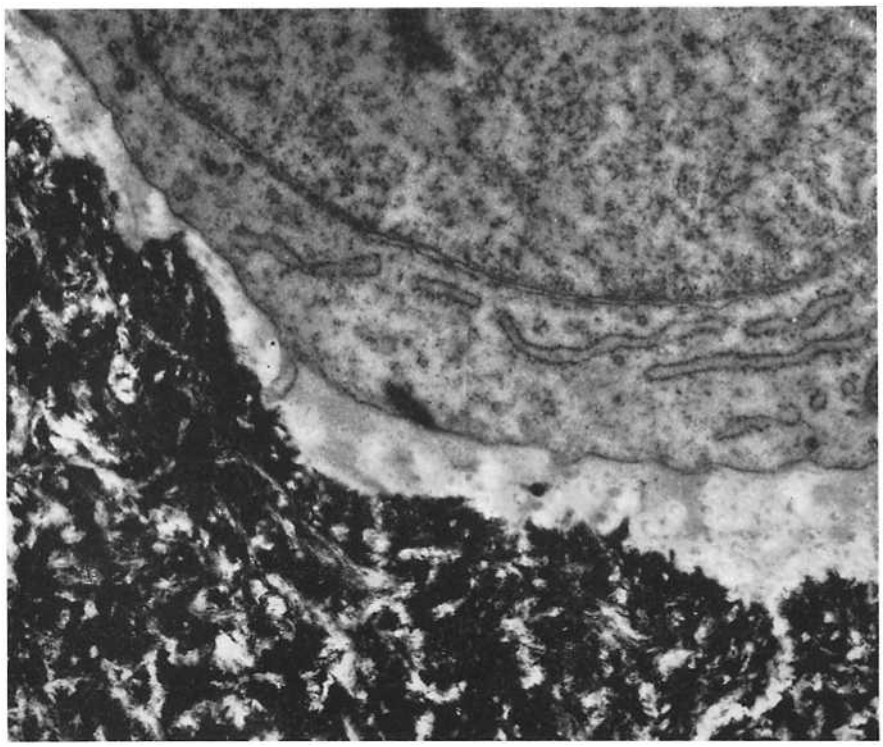

FIGURE 3 Part of an osteocyte showing both nuclear and cell membranes. The cell is separated from the bone matrix by an uncalcified osteoid border which contains sections of collagen fibrils. The bone matrix is seen as a dense mass of needle-like crystals of calcium apatite. Section cut at 400 A and floated for about 3½ minutes on *aq. dest.* × 25,000, Kodak grade 4 paper.

encourage rapid drying, and so probably less than 0.01 gm of fluid would remain to be evaporated.

In most laboratories, the pH of distilled water is not at the point of neutrality (pH 7), but is usually between pH 5.2 and 6.0, owing to the rapid absorption of carbon dioxide from the atmosphere. However, by its method of preparation, the solution of calcium phosphate is saturated, with respect to both calcium and phosphate ions, at the prevailing pH of the distilled water. Under these conditions, the actual pH of the distilled water does not matter; in this laboratory the pH of distilled water is about 5.4, and the saturated calcium phosphate solution 6.1.

Table I gives the data for two tests (Nos. 7 and 8) using sections collected on calcium phosphate–saturated water. A difference between sections with low and high T_s values was not detectable, all sections appearing fully calcified with no evidence of decalcification, despite floating for 30 minutes after sectioning. This latter fact lends strong support to the hypothesis that the bone mineral is removed by the solvent action of water rather than by the other mechanisms previously stated. Also, the fact that sections appear fully mineralised when collected, as soon as possible after sectioning, onto distilled water (in less than 5 minutes) and begin to appear demineralised only after 6½ to 8 minutes argues further against the removal of crystals otherwise than by solution. Grids loaded with undecalcified sections have been subjected to rapid shaking by attaching them to an arm vibrating at about 3000 cycles per second for 5 minutes in an attempt to shake off the crystals, but no loss of crystals was observed.

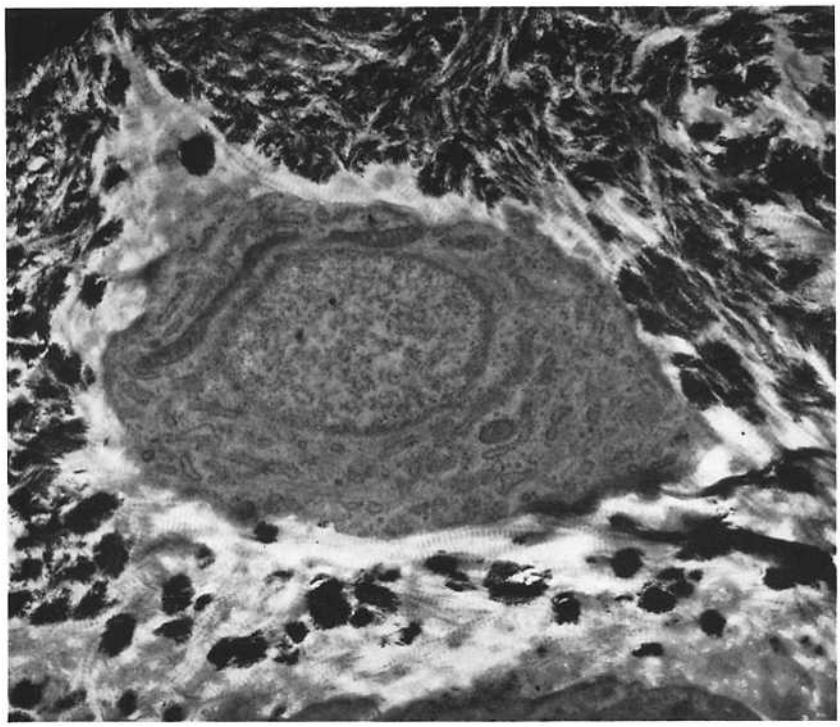

FIGURE 4 An osteoblast being incorporated into the bone matrix. Striated collagen fibrils of the preosseous matrix are evident near the cell. The bone matrix has the typical appearance of well mineralised bone. Sections cut at 500 A and floated for about 3½ minutes on *aq. dest.* × 12,500, Kodak grade 4 paper.

It seems likely, therefore, that the decalcification which occurs in thin sections of bone after they have been allowed to float on distilled water for more than 6½ minutes is caused by dissolution of the bone crystals rather than by mechanical stress acting during manipulation of sections prior to examination in the electron microscope.

The use of calcium phosphate–saturated water

as a collecting fluid prevents decalcification, but, at present, it is an open question whether any calcium phosphate crystallises onto the bone. No sign of crystals has been seen on sections of osteoblasts or osteocytes, but nucleation centres are, presumably, not present within cells. Since osteoclasts may already contain crystals, the problem of recrystallisation is more difficult. However, the crystals only occur in the ruffled border region of the cell, suggesting that their presence is due to cell activity rather than to recrystallisation.

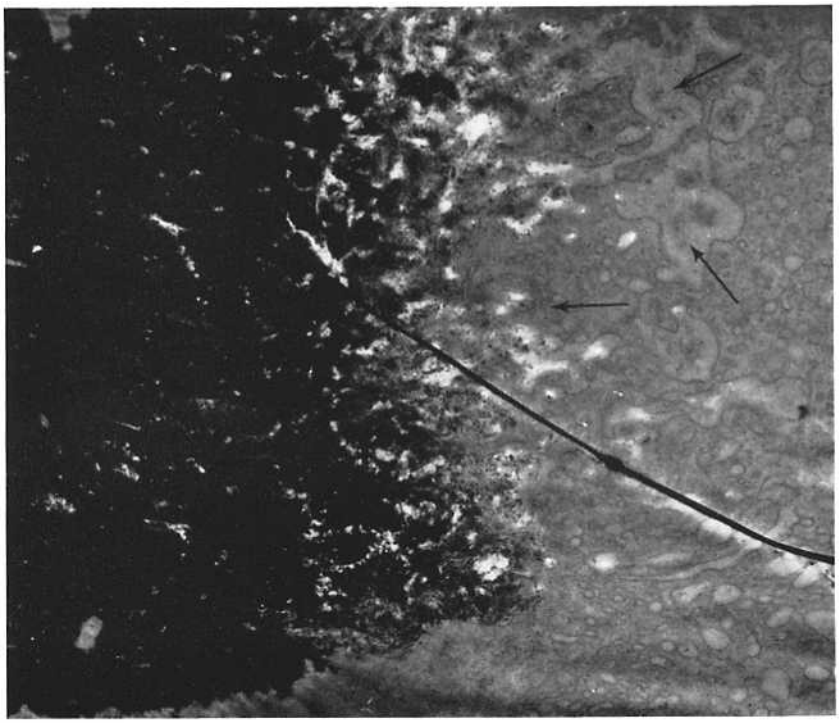

FIGURE 5 Active site of an osteoclast. The ruffled border folds contain crystals of apatite (arrows) removed from the bone matrix which itself is solid and dense in appearance, indicative of full mineralisation. The diagonal black line is a fold in the section. Section collected on calcium phosphate–saturated water. × 15,000, Kodak grade 4 paper.

A feature of decalcified sections is the persistence of crystals at the edges of bone trabeculae underlying active bone-modelling cells. This is not seen in the matrix under inactive cells where crystals are removed up to and including the bone edge. Usually a much greater depth of retained crystals is found under the active sites of osteoclasts than under osteoblasts, and often crystals remain within the folds of the osteoclast's ruffled border (Fig. 2) and its pinosomes. The reason why these crystals remain within the section may be derived from a comparison of crystals in the environment of the edge with those in the centre of bone trabeculae. In the latter instance, the crystals are closely packed, being surrounded by other similar crystals. Near osteoblasts, crystals are in the process of formation and as such will vary in arrangement from the cell edge, where discrete crystals and small aggregates are found, to a certain depth inside the bone where crystals are closely packed and of fairly uniform size. A similar pattern holds for the region near the osteoclast where, however, the bone is being progressively eroded, the depth of activity of the cell in the bone being greater than that of the osteoblast. In both cases, the embedding medium is probably able to penetrate the tissue by replacing the extracellular fluid which normally bathes these

regions, and so surround the looser more widely spaced crystals with a matrix of water-insoluble material, but it is not able to penetrate the compact formed bone because in these "fully calcified" regions any remaining osteoid water is "bound" (Robinson, 1960) and not replaceable. When the material is cut into sections, the impermeable

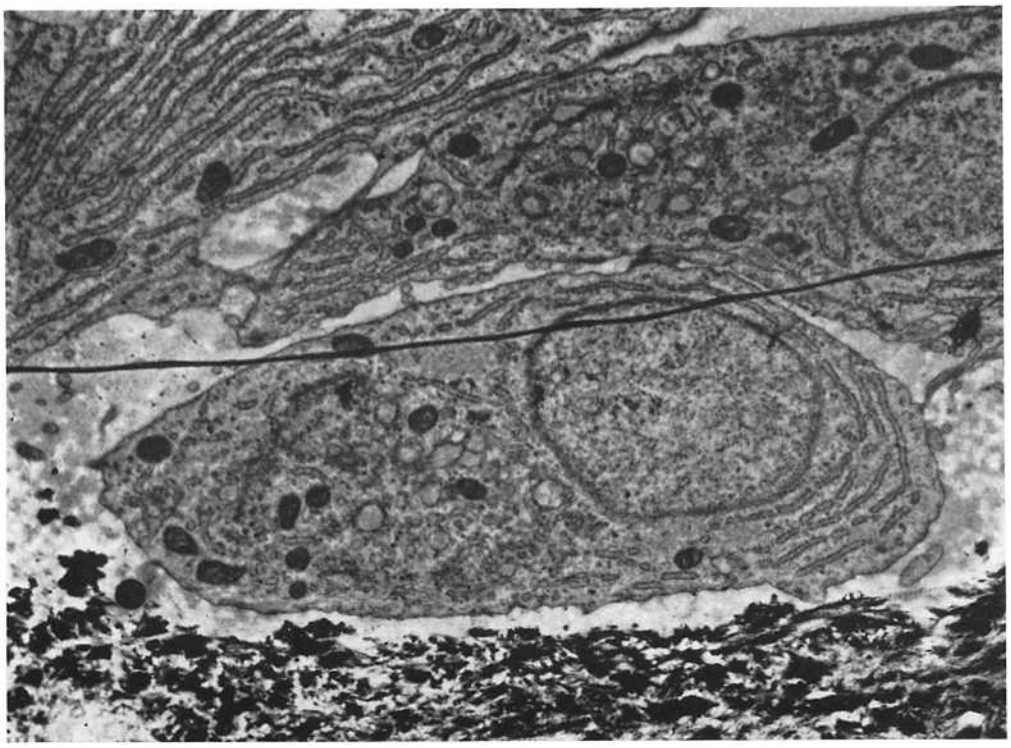

FIGURE 6 Parts of three osteoblasts and adjacent bone. One cell is separated from the bone matrix by an uncalcified osteoid border. Compare the enhanced contrast of the cellular detail with that of Fig. 5. The black line across the figure is a fold in the section. This section was collected on calcium phosphate–saturated water and stained by the author's modification of the lead acetate technique of Dalton and Zeigel. × 12,500, Kodak grade 3 paper.

Araldite retains crystals near active cells, but water from the collecting and staining baths dissolves crystals in the deeper parts of bone spicules not so coated. This effect has been noticed in preparations infiltrated by and embedded in either Araldite or Borysko's (1954) prepolymerised methacrylate syrup, which are highly viscous liquids. It is not known whether methacrylate monomer is able to penetrate bone and thus prevent demineralisation throughout the thickness of bone spicules. Probably the defects of methacrylate monomer embedding and subsequent polymerisation, such as explosion artefacts and poor cytologic detail, are not worth the possible advantage to be gained of crystal protection.

There seems to be no reason why the technique, described in this paper, to prevent dissolution of bone crystals in lead acetate stain should not be extended to other stains for electron microscopy, provided no interaction takes place. It is likely to be of value in conjunction with uranium acetate, the alkaline lead stain of Karnovsky (1961) and of Lever (1960), and other similar stains, although these have not been tested during this work. It is hoped to carry out such tests in future studies.

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