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Harold M. Frost

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# OBSERVATIONS ON PASSIVE TRANSFER OF BONE MINERAL IN MAN AND NUCLEATION

HAROLD M. FROST, M.D.

## INTRODUCTION

Much work and writing in the last five years indicates that mineralization of human bone matrix is probably a biphasic process. The theory is as follows:

1) The first phase is known as nucleation. As summarized by Glimcher<sup>9</sup> and Neuman,<sup>11</sup> nucleation is the formation of the nucleus or base of a crystal in the bone matrix. The nucleus involves a few of the ions characteristic of the hydroxyapatite lattice, depends on specific chemical and steric attributes in the matrix, and follows some specific but unknown chemical "maturation" process in newly formed matrix. The formation of the nucleus may involve an organic epitaxy.

2) The second phase of bone mineralization has not been named. Here it will merely be called mineralization to distinguish this phase from the nucleation phase described first.

Bone mineralization in normal body fluids proceeds independently of the action of a living cell because, in terms of activity coefficients, the body fluids are supersaturated with respect to the mineral crystallites. The presence of preexisting hydroxyapatite crystals seems to be all that is needed for the deposition of additional crystals.

3) The ability to calcify is characteristic only of certain tissues in the body. These tissues are teeth, hyaline cartilage, and bone. The specific feature or features which permit these tissues to mineralize are to be found in the cement substances of each tissue; the collagen appears to be structurally and chemically the same in all three and similar to the collagen in the remainder of the body's non-mineralizing tissue.<sup>9</sup>

In this paper certain simple observations are presented which bear to some extent on the theoretical model just outlined. The observations are fortuitous rather than experimental.

## OBSERVATIONS AND DISCUSSION

### PASSIVE TRANSFER OF MINERAL

In this laboratory fresh, undecalcified bone sections are the major investigative material. The sections are made and stained by methods which produce less artifact in sections than is produced by any other existing sectioning method.<sup>3,4</sup> Human biopsy specimens prepared by these methods have been kept alive for several weeks in tissue culture.

When fresh, wet undecalcified, unfixed, unembedded human bone is stained with 1% basic fuchsin in 40% ethanol, the resulting stain reveals two things. First, all holes normally found in bone are stained red.<sup>1,4</sup> Second, and more important for the present purpose, all bone mineralized below about 80% of its maximum is fuchsin permeable, and thus a diffuse red similar to an automobile tail light roundel. All

bone mineralized above 80% (this is only roughly measured) is impermeable to the stain and appears as clear and colorless as clear glass on finished sections. The holes in the bone are clearly visible.<sup>1,4</sup>

With the aid of these methods a new bone disease termed feathering<sup>1</sup> has been discovered. (Fig. 1) Feathered bone is incompletely mineralized morphologically, and markedly delayed in its rate of further mineralization dynamically. Some skeletons contain impressive proportions of feathered bone.

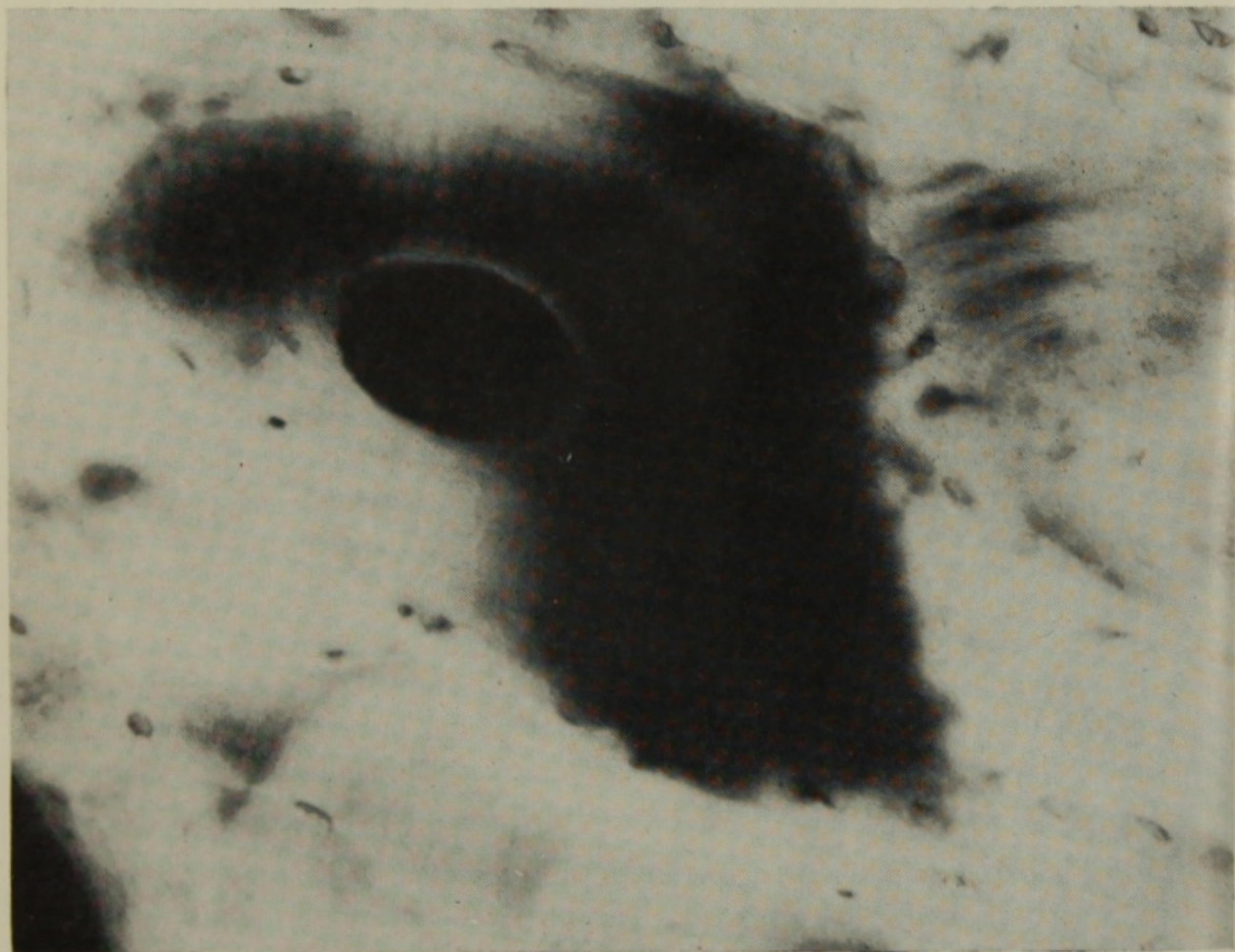


Figure 1

Fresh, undecalcified human clavicle about 200 x, cross section, basic fuchsin, Wr. 58. The Haversian system in the center is feathered. The black portion is heavily stained with fuchsin due to low degree of mineralization. A clear, highly mineralized ring lines the Haversian canal. The disease stops at the cement line.

There are several cases in the case material obtained by the laboratory with very severe feathering revealed by the initial sections. Over 9 months after these specimens were obtained, and after the initial fresh sections had been prepared, it was decided to make an additional batch of sections for resident teaching purposes. The new sections were prepared from bulk material such as rib or clavicle that had been stored at room temperature in 40% ethanol.

The new sections contained markedly less fuchsin permeable bone than the original sections in every one of the four cases in which this phenomenon was sought for.

## Bone Mineral and Nucleation

We must infer that during the months of shelf life, mineral in areas of high mineral density redistributed to areas of low mineral density within the bone. There was no outside source of mineral; the specimens were kept in glass jars with air tight seals.

This simple observation means several things.

A) In vitro, areas with low mineralization per unit volume of bone possess lower free energy than areas with high mineralization per unit volume of bone. The transport of mineral observed in vitro must have occurred at the expenditure of energy falling down a gradient which originally existed in the bone. This will be recognized as a thermodynamic concept.

B) If considered as an isolated non-living system, wet bone that is undamaged by embedding, fixation, heating, or the like tends to equilibrate the continuous spectrum of mineral densities normally present in a bone.

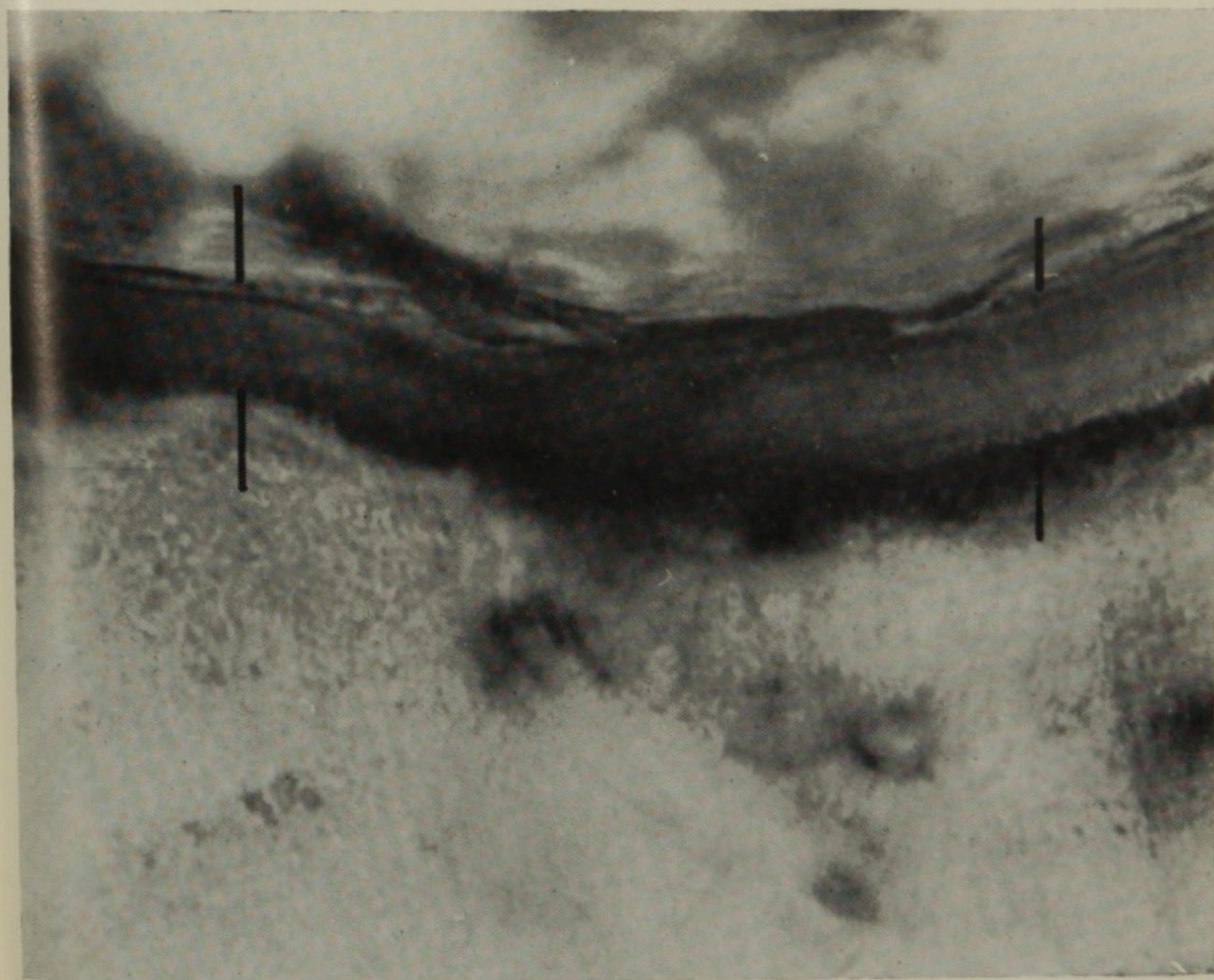


Figure 2

An osteoid seam as revealed in fresh, undecalcified sections. The seam lies between the bar markers. About 500 x. The upper edge of the seam is the wall of the vascular channel. The lower edge of the seam is advancing wall of mineralization referred to in the text. The lower half of the figure is mineralized bone. The direction of growth and of mineralization is upwards.

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C) The presence of an unstable energy gradient at the time the bone samples were placed in the jar for storage implies that the gradient was originally created

by living cells. This in turn implies that during life the cells maintain such gradients. At present there would seem to be as much reason to expect the cell to create zones of low free energy as there is reason to expect the cell to create zones of high free energy. In other words, the bone cells could either be pumping mineral into bone against a gradient, or pumping mineral out of bone against a gradient. These are the two possible situations which reason and data must eventually permit us to choose between.

D) In vitro in intact bone in an isolated system, progressive mineralization of already partially mineralized bone occurs.

#### PROBABLE SITE OF NUCLEATION AND MATURATION IN HUMAN BONE IN VITRO

Previously reported work recorded the fact that human lamellar bone normally forms first as an osteoid seam laid down on preexisting bone. Osteoid seams are not usually an abnormal feature of human bone, as is often taught, but are a normal

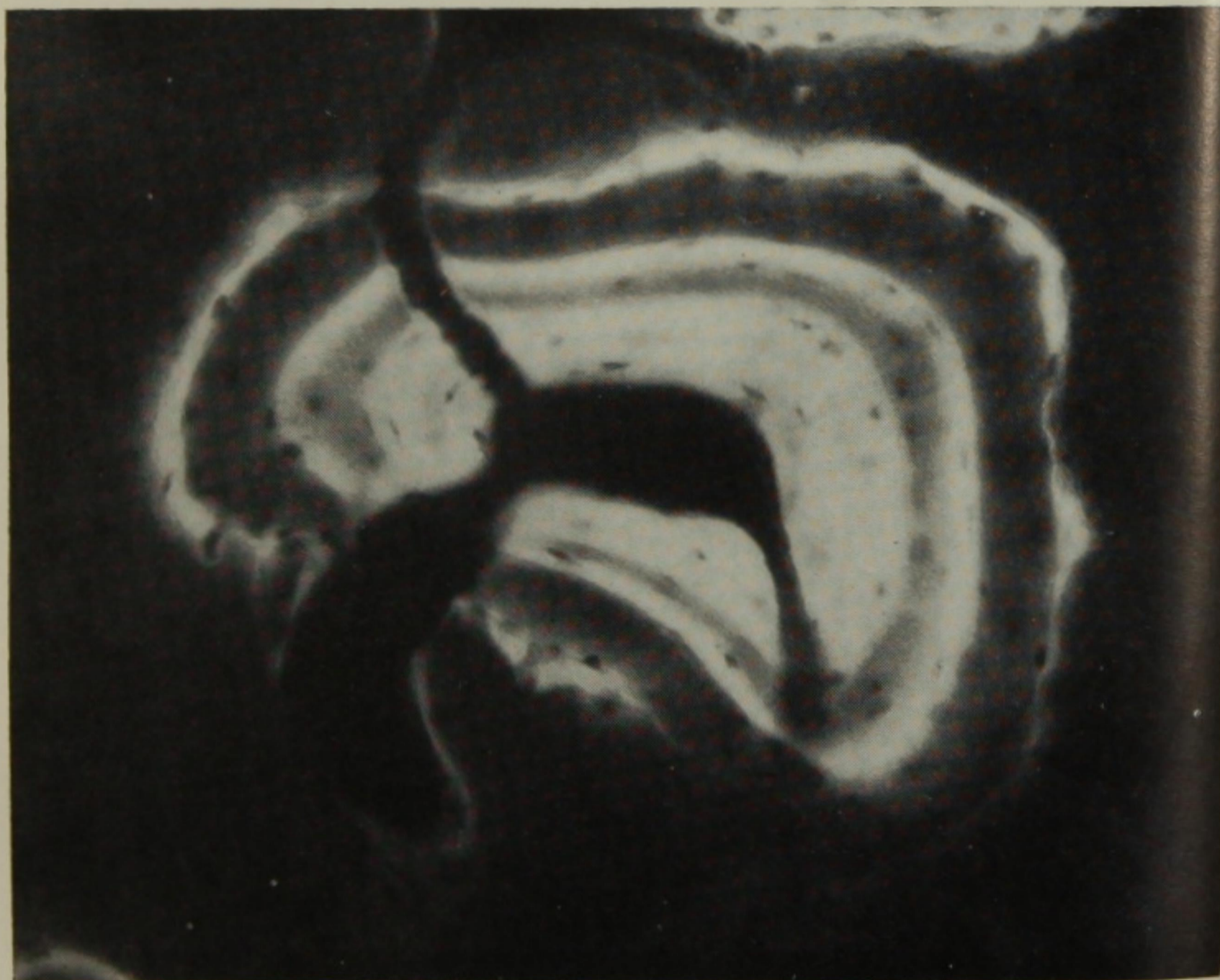


Figure 3

Cross section femur 48 year old man, fresh, undecalcified. Urinary tract infection 1956, treated with tetracycline intermittently. Amputation 1960 for tibial neoplasm. The bright bands are tetracycline, fluorescing with the writer's microfluorescence set-up, in an Haversian system. Each band represents a different time and duration of drug dosage. When the times and durations are known, the remodelling rate 4 years before amputation can be measured. New osteoid is deposited at about 1 micron per day. It is mineralized at the same rate. The tetracycline is deposited and in some manner fixed in situ where the osteoid begins to mineralize.

## Bone Mineral and Nucleation

accompaniment of normal growth and normal skeletal remodelling.<sup>2,6,10,11,12</sup> Their presence in abnormal numbers, and in abnormal widths, are abnormal, but this is a separate story. (Fig. 2).

The simple facts above mean this: the only places in the lamellar bone skeleton of the human child and adult where the nucleation process described earlier occurs is in osteoid seams. The remainder of the skeleton is by definition, and in fact, already mineralized to varying degrees and so has already passed through the stage of nucleation. *Therefore, to study nucleation in human bone, osteoid seams must be studied.*

While much has been written about osteoid seams from the standpoint of osteomalacia, little has been written about their normal evolution and change. Here however the tetracycline bone labelling phenomenon reveals a great deal about human osteoid seam physiology in vivo. (Fig. 3).

The tetracycline bone labelling phenomenon is discussed at length elsewhere.<sup>7,8</sup> In brief, any of the tetracycline antibiotics present in the blood are deposited on all available bone surfaces and in the peripheral zone of osteoid seams where mineralization is beginning. When the tetracycline is discontinued, the surface stain in the skeleton disappears within 48 hours. The tetracycline deposited at the periphery of the osteoid seam where mineralization of osteoid commences remains permanently in place, the maximum observed time being 9 years as of this writing. The tetracycline fixed in the mineralizing osteoid does not diffuse in vivo or in vitro in fresh, undecalcified sections. The skeletally fixed tetracyclines can be detected and measured with the aid of a simple microfluorescence set-up.<sup>5</sup>

*Any new bone forming during the administration of a tetracycline antibiotic is labelled by the tetracycline.* It remains in situ until resorption of the bone occurs. Accordingly it is possible to measure the amount of new bone formed in unit time in a unit volume, and to observe where this activity occurs and is most or least active.

The reader will recognize a powerful, new and economical technique for investigation of human bone physiology in vivo. This possibility, long a dream, is now reality.

Observation of an extensive amount of human tetracycline labelled material has brought to light the following facts pertinent to the present presentation:

- A) A small arbitrary unit volume of osteoid is formed by an osteoblast.
- B) The unit volume of osteoid exists as osteoid for about 10 days, during this time being buried by additional new osteoid deposited each day.<sup>6,8,12</sup>
- C) At about the 10th day mineral suddenly appears in the unit volume of osteoid. In a 4 day period about 75% of the total amount of mineral it can ever hold is deposited. The remaining 25% of the total possible deposit of mineral is deposited at an exponentially slowing rate for the remainder of the biological life of the matrix.
- D) Under the light microscope an osteoid seam in an Haversian system appears as a ring of osteoid lining the Haversian canal, and thus separating the canal from

the mineralized bone. The periphery of the osteoid seam is the zone of osteoid which is about 10 days old, which is rapidly mineralizing, and which is creeping centripetally towards the center of the Haversian canal at the same rate as new osteoid is added each day by the osteoblasts lining the inner wall of the seam.

On the basis of the above facts the following may be inferred:

The mysterious chemical maturation in the osteoid seam which permits mineralization, and which is supposed to lead to nucleation, must occur either in the zone of initial mineralization (which is about 4 microns thick) or slightly in advance of this zone. The nucleation process in human bone, if it occurs (and there is good although not compelling evidence to support its existence), must occur at the inner surface of the advancing wall of mineralization at the periphery of the seam.

Therefore, if maturation of the matrix and nucleation are to be studied in human bone, attention should be directed to the normal osteoid seams of children and adults. Attention should be limited to lamellar bone because fibrous bone formation is not normally found in healthy humans.

The fact that tetracyclines are permanently deposited only in newly formed, progressively mineralizing lamellar bone (actually also in mineralizing fibrous bone and hyaline cartilage), and not permanently deposited in any other part of the lamellar skeleton, even though it be permeable to tetracyclines in vivo, strongly suggest that the mechanism of permanent fixation is associated either with the maturation process, or the nucleation process, or both. This means that the tetracycline labelling phenomenon may be the key needed to unlock the secrets of the maturation and nucleation processes.

#### SUMMARY

1) Equilibration of mineral densities in dead human bulk bone in closed systems over several months has been observed. The cause of the phenomenon is conveniently described in thermodynamic terminology.

2) Knowledge of human osteoid seam physiology indicates that the chemical maturation of bone matrix and the nucleation process leading to mineralization of human lamellar bone probably occur at the periphery of osteoid seams. Study of the peripheral zone, which is 4 microns wide, would yield more information about the maturation and nucleation processes than study of the remainder of the skeleton. The key to the study of the maturation and nucleation processes may be tetracycline fixation in the maturing and mineralizing osteoid.

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