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# HALO VOLUME*

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This paper presents evidence for the existence and dependence on osteocyte metabolism of a feature existing in perfectly fresh undecalcified bone sections and first described by Frost in 1957.[8] The feature is termed halo volume because it exists in three dimensional space as a "halo" around living osteocytes and adjacent parts of their canaliculae. H. V.'s (halo volume's) most important feature is a quasipermeable state of the bone wall of lacunae and canaliculae. It is normal characteristic of fresh bone.

The majority of the evidence presented was elucidated by us but additional and prior evidence from other laboratories exists and will be presented briefly.

## MATERIALS

Bones from over 100 humans of all age decades and both sexes plus bones from over 30 dogs, sheep, monkeys, rats, rabbits and mice have been examined by the techniques which will be mentioned. In the majority of the cases the cortex of long bones was examined but cancellous bone from metaphyses and axial skeleton have also been examined.

## METHODS

Undecalcified, fresh bone sections are made and stained for H. V. by Frost's techniques.[3,4] These techniques have the important requisite of permitting examination of bone with introduction of less artifact than other existing methods. Several peculiar aspects of H. V. demonstration will be briefly mentioned.

Bones must be examined no more than 6 hours after death or removal at surgery. During this time drying must be prevented by immersing bulk material in tap water, distilled water, normal saline or Ringer's solution.

The following treatments consistently cause the majority of H. V. to disappear and the remainder to be smaller than normal in experimental sections as compared to control sections kept in distilled water at room temperature: Immersion in methanol, ethanol, propanol, isopropanol, acetone, dioxane, glycerine, formalin, heavy metal fixatives, solutions with unphysiologic pH's or molarity, solutions containing ions which form insoluble precipitates with bone salt ions; freezing; refrigeration above freezing; drying for over 30 seconds; storage longer than 6 hours. It is astonishing that fresh material should contain a feature so sensitive to such a number of procedures in common histopathological use. We believe this explains why H. V. have escaped prior notice.

Frost's paper details several methods for staining H. V.[4] The permanganate method is the simplest, quickest and has been completely reliable in our hands. In brief, a freshly made section is boiled 12 minutes in 0.1 N $KMnO_4$. Grind off all resulting

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stain from the bottom and part from the top of the section. Dry on a warming plate and mount in neutral synthetic resin. The mount is permanent. Section thickness is immaterial. Only the surface 10-20 u are stained, the remainder of the section serving as a mechanical carrier for the stained portion.

The following orientation is given for the reader unfamiliar with stained, undecalcified sections.

Since no definition of normal or abnormal degree of mineralization exists, we have adopted Frost's arbitrary standard of permeability to 40% alcoholic basic fuchsin. "Normally' mineralized bone is not permeable, "incompletely" mineralized bone is permeable to this reagent. Numerous other histological dyes tested behave in the same manner as basic fuchsin.[4]

Normally mineralized bone stained with these reagents reveals unstained interlacunar and intercanalicular bone, stain on the walls of the physiologic spaces in bone and stain of the protoplasmic contents of these spaces. Failure to grind off surface stain prior to mounting has led to the erroneous and widespread impression that diffuse permeability to the organic histological dyes exists in undecalcified sections.

Within the inherent optical limitations (0.95 N. A., $\pm$ 0.3 u resolution) the size of lacunae and canaliculae in fuchsin stained and in air mounted sections is the same.

## HALO VOLUME DESCRIPTION

H. V. is the 0.5 — 2.0 u depth of bone wall of the lacunar and canalicular lumens. This part of the bone wall normally is permeable to small but not to large ions and is stained by the recommended methods. This selective permeability is the major observable feature of H. V. Because the recommended permanganate stain penetrates into the bone wall of the lumens, the lacunae and canaliculae appear larger in H. V. stained sections than in fuchsin stained sections from the same case. (See Fig. 1, 2).

With the permanganate method outlined, H. V. have been demonstrated in all of the material listed under Materials. One's immediate reaction is that this is artifact resulting from the elevated temperature and from the fact that the lumens are the easiest access routes for the stain reagent.

The suspicion of artifact is dealt with by the 11 observations next presented.

1. H. V. is demonstrated by other techniques using room temperature, different reagents and with anionic and cationic reagents. Some substance(s) may be selectively leached out of the H. V. moiety of bone without affecting the remainder. Blocking phenomena in H. V. can be produced.[4]
2. No H. V. stain pattern results with the large moleculed dyes tested with the single exception of sodium alizarin sulfonate. This is true at room temperature and at 37° C.
3. Control sections of fresh, live bone invariably reveal H. V. with the per-

manganate technique recommended while experimental sections variously treated as listed under Methods lose the majority of the H. V., the remainder being smaller. The only exception has been bone from a case of vitamin D resistant rickets whose very large H. V. were not noticeably affected by alcohol or storage time.

The above behavior suggests some physical change in the H. V. bone moiety as a result of the described treatments. As a result of this change, small ion permeability is drastically reduced in H. V.

4. Micropetrosis and osteocyte death in vivo have been discussed in other papers by Frost,[6,7] by Jaffe and Pomerans[10] and by Sherman and Shelakovitch.[14]

   (a) Micropetrotic bone is dead. H. V. cannot be stained in micropetrotic bone.

   (b) There are usually fewer live osteocytes in extraHaversian bone than in Haversian. There are usually fewer H. V. stained in extraHaversian bone than in Haversian.

   These facts suggest that a live osteocyte is a requisite for H. V.

5. No. H. V. can be stained with the recommended techniques in sections decalcified, prior to staining, in dilute nitric, hydrochloric or acetic acids or by citrate complexing. No H. V. stain is seen in routine H&E bone preparations.

6. The parts of canaliculae up to about 20 u from the osteocyte show H. V. stain but the parts farther away normally do not, in spite of the fact that generically all parts of canaliculae are exposed to the section surface at one or another point. The silver and lead sulfide methods are occasional exceptions to this rule and sodium alizarin sulfonate a frequent one.

   Again some physical difference between H. V. staining and non-H. V. staining bone must exist to explain this difference in behavior.

7. Fig. 1 is a photomicrograph of a fuchsin stained cross section from a 72 year old man dying of gastric hemorrhage. Fig. 2 is of a permanganate stained section of the same bone at the same magnification. Fig. 3 is another permanganate stain done after storing the bone 48 hours in 40% ethanol. The apparently larger size of the lacunae and canaliculae in Fig. 2 as compared to Fig. 1 is evident. The loss of H. V. in Fig. 3 following storage is equally evident. Less than one minute in alcohol is needed to initiate this difference.

8. Fig. 4 illustrates the H. V. in a control rat immediately after sacrifice. Fig. 5 illustrates the H. V. in a littermate given 1000 units of parathormone parenterally 12 hours before sacrifice. The H. V. are more numerous, larger and affect a greater length of the canaliculae. Some areas in Fig. 5 are micropetrotic and are free of H. V.

9. Fig. 6 illustrates a CoS stain on a section from a boy with vitamin D resistant rickets. The specimen was obtained at osteotomy through the courtesy of

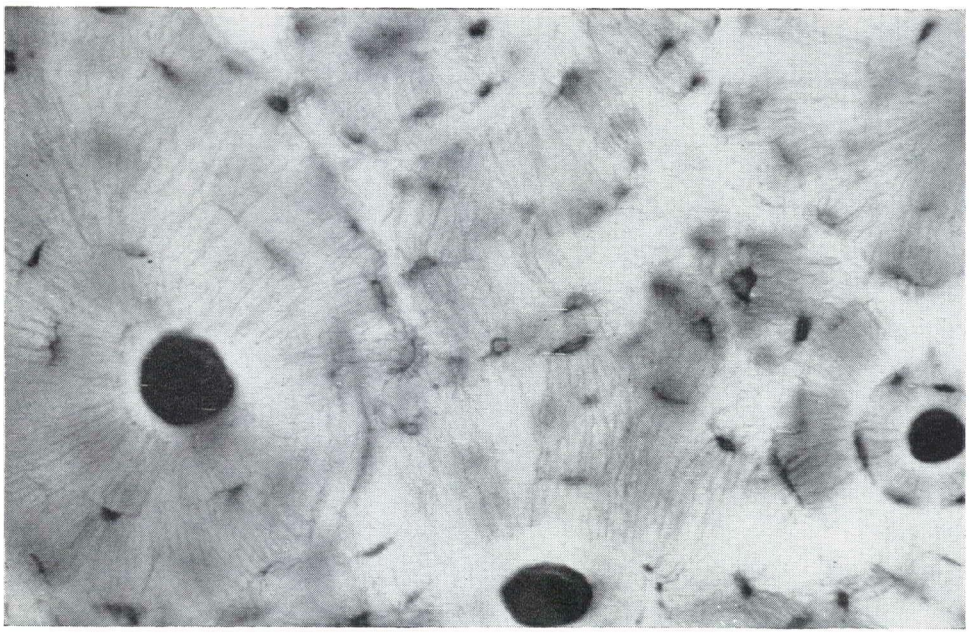

Figure 1

Cross section clavicle 72 yr. male. 0.95 N. A., 400X. Stained with basic fuchsin. The dark band around the osteocyte lacunae is fuchsin and represents the true size of the lacunae. The canaliculae are barely resolved.

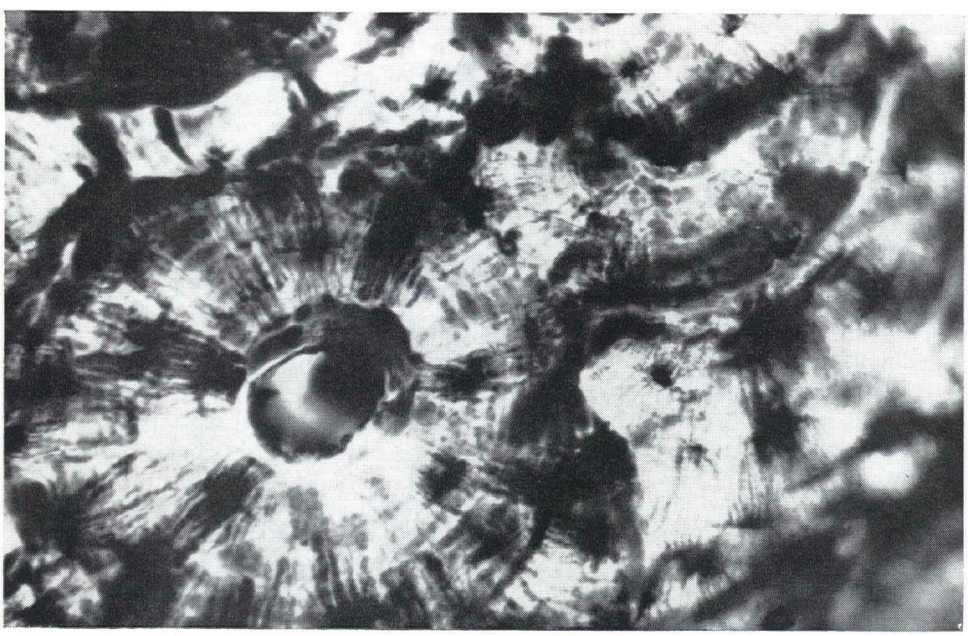

Figure 2

Cross section same bone, same optical factors. Stained with permanganate as recommended in the text. The "spiders" in the Haversian system to the left and below the center are halo volumes. The permanganate has penetrated into the bone wall around the lacunar and canalicular lumens giving the impression they are a great deal larger than they actaully are. Compare with Fig. 1.

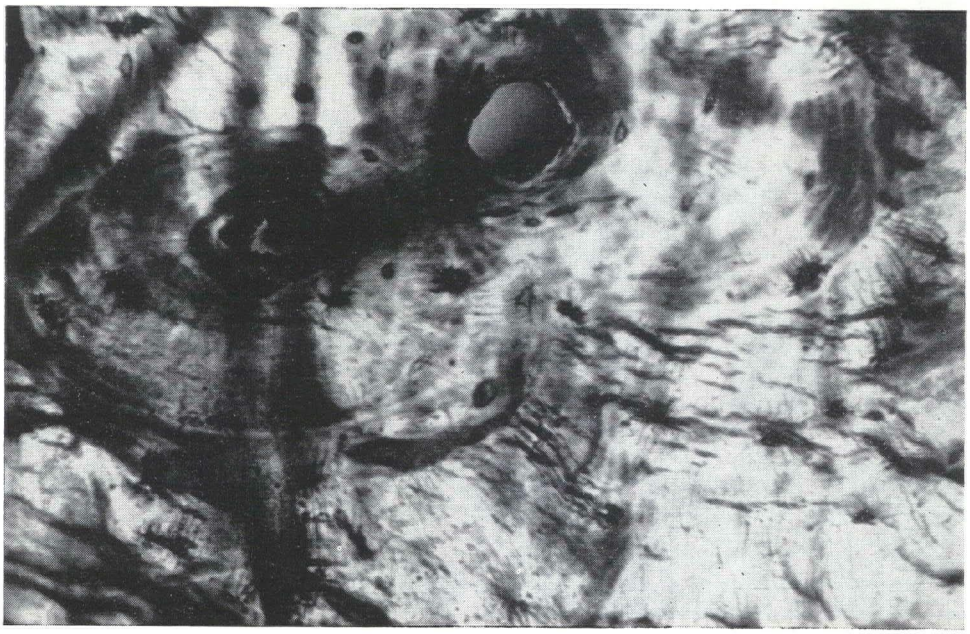

Figure 3

Cross section same bone, permanganate stain but done two days after death of patient. The storage time has caused the permeability in the H. V. which was present in the perfectly fresh material (see Fig. 2) to disappear for the most part. The vertical markings to the left of center are surface stain left on to prove that the difference is not due to grinding off more material in this section than in that of Fig. 2, which actually had more surface removed than the present section.

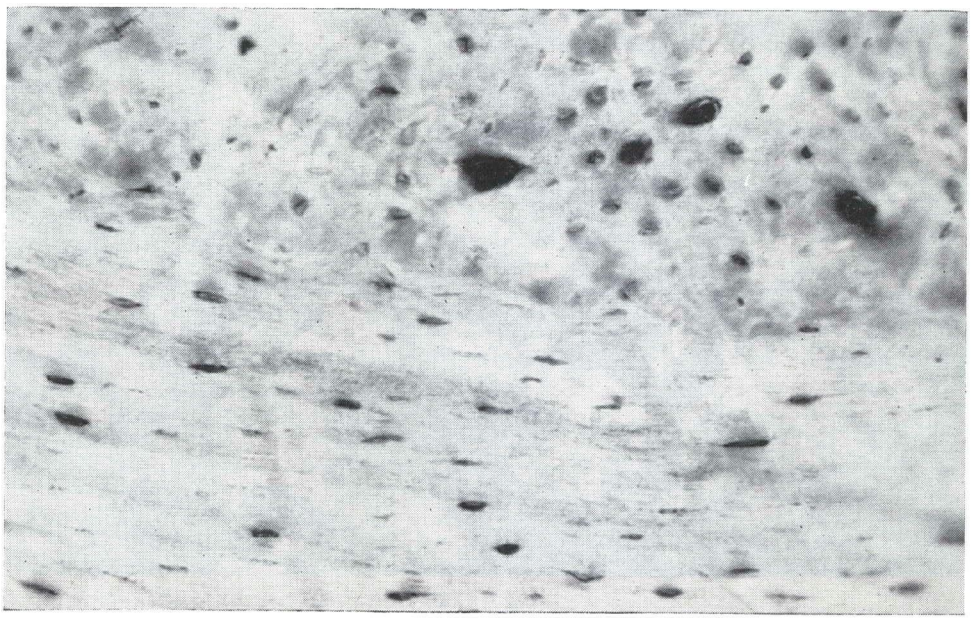

Figure 4

Cross section M/3 femur control rat, permanganate stain. Same optical factors as in Figs. 1-3. Very poor stain for H. V. Vertical striations are identification of surface grinding marks as in Fig. 4.

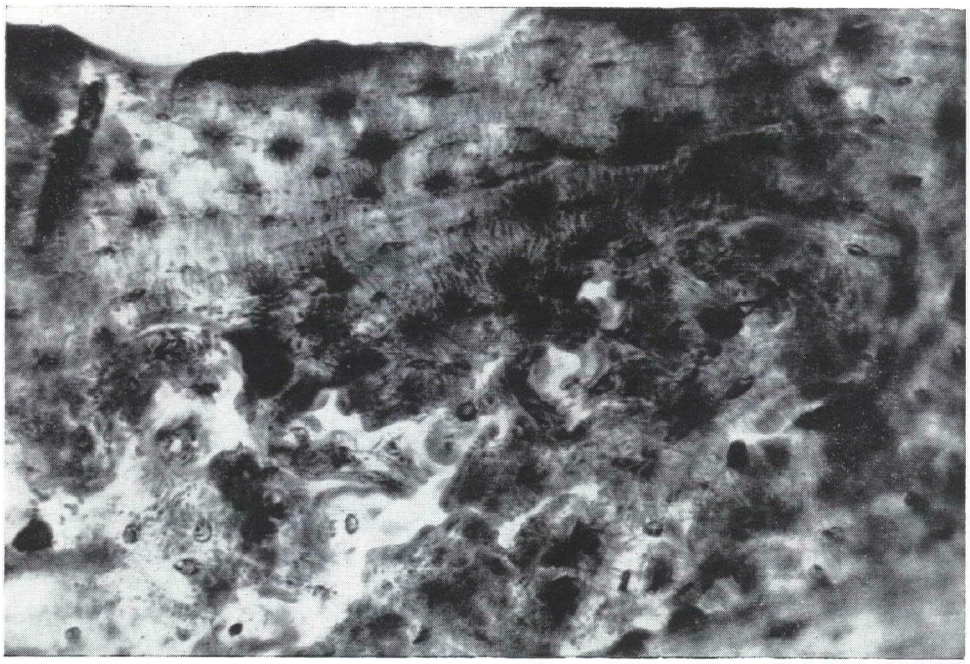

Figure 5

Same stain and optical factors but rat given 1000 units parathormone 12 hrs. before death. Large H. V. around lacunae and canaliculae. The clearer bone below and to left of center is micropetrotic.

J. D. Fleming, M.D. The large, black blurs represent markedly enlarged H. V. Fig. 7 illustrates a fuchsin stained section from the same case. The dark blurs represent fuchsin permeability around osteocytes. Fuchsin permeable H. V. are rare in the experience of this laboratory. Further details of this case and the findings are published.[5]

10. Examination of fresh, unstained sections in water mounts with the Baker A-0 interference microscope reveals that there is a shorter optical path in the H. V. bone compared to the surround. The volume of bone so affected is usually larger by a factor of about 3 than that stained with the permanganate method. After drying and mounting in synthetic resin most of the H. V. detectable by interferometry disappear, the remainder becoming smaller than they previously were.

    Dilute, water soluble stains such as methylene blue may be used to permit identification of lacunae which are empty of protoplasm in water mounted sections. Since the time required for staining beyond the surface 10 u of the sections is too long for preservation of H. V., this method is confined automatically to examination of lacunae close to the surface of the section. Empty lacunae do not usually manifest H. V. and full ones usually do. There are a certain number of "sports, varying from 0.5-3.0% which violate the above rule.

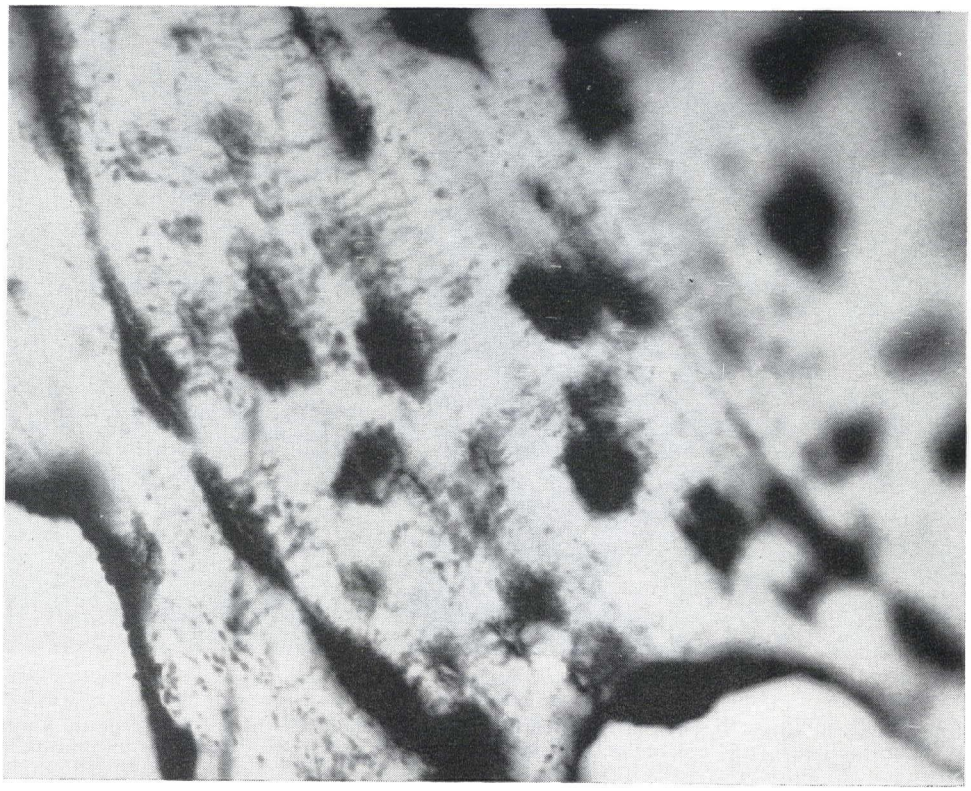

Figure 6

Colbalt Sulfide stain tibia 9 yr. male with vitamin D resistant rickets. 0.95 N. A. 600 X. The large black blurs are enlarged H. V. A few much smaller H. V. are interspersed which give some idea of the true lacunar dimensions.

Interferometry is an important confirmatory technique for the thesis of this paper since the image produced in the interference microscope depends on an inherent difference in refractive index among the various components of the object. The staining techniques depend on permeability and if this were our sole evidence, suspicion of artifact would be justifiably entertained.

11. Initially we mentioned that other authors have published evidence for existence of H. V.

    (a) Heller-Steinberg published fascinating work in 1951 done on fresh, undecalcified frozen-dried bone.[9] With a silver technique, PAS and toluidine blue she was able to produce, describe and illustrate enlarged H. V. in a group of rats given parathormone as compared to a control group. She speculated that the parathormone might have exerted a regulatory effect on the osteocyte metabolism. Silberberg and Silberberg later duplicated successfully parts of Heller-Steinberg's work.[15]

    (b) Several authors have published high resolution microradiographs of bone from cases of vitamin D resistant rickets. The current example is

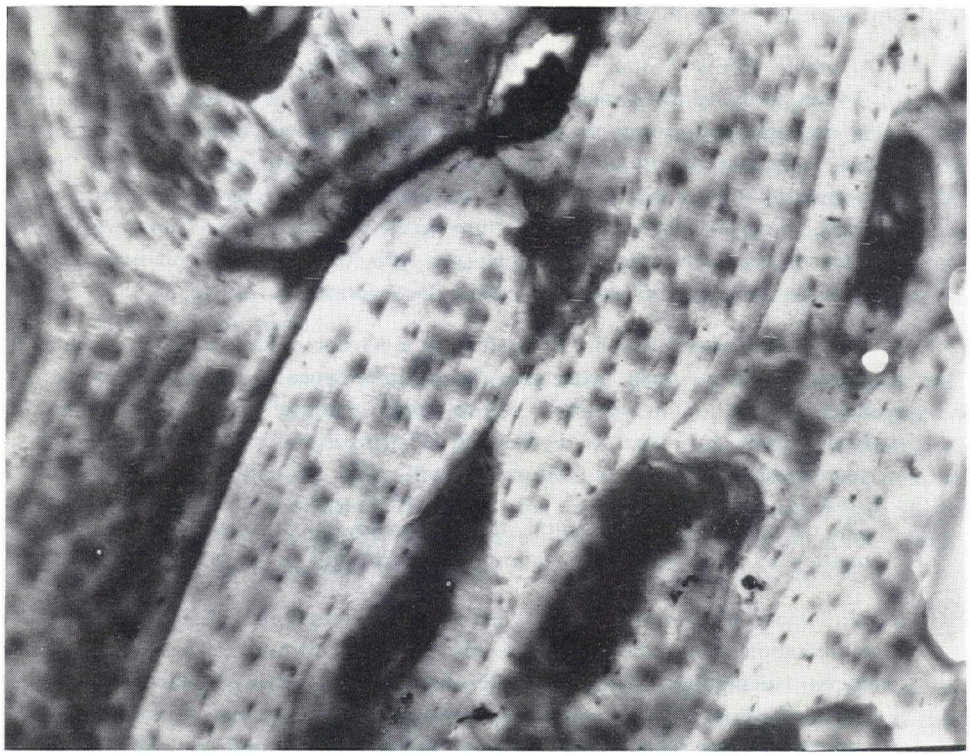

Figure 7

Fuchsin stained section from same case as in Fig. 6. 0.65 N. A. 200X. The very dark, broad vertical striae are volumes of fuchsin permeable bone. The small dark blurs scattered over the field are enlarged H. V. which are fuchsin permeable. On the original some canaliculae are resolved and the osteocyte nuclei can be seen as tiny, very dark dots in the center of lacunae. Where fuchsin permeates into the bone mineralization is defective in amount per unit volume. As a result microradiographs of vitamin D resistant rachitic bone also reveal a dark blur in the H. V. due in this case to lack of obstruction of the x-ray beam.

the paper by Engfeldt and coworkers.[2] In their illustrations the radiolucent H. V. around many of the osteocytes present is manifested as a dark blur very similar to Fig 7 in this paper. Their imagery depends on physical absorption by calcium nuclei of gamma rays from a monochromatic source while ours depends on permeability to an organic dye. The convergence of findings with such different methods is striking to us.

## DISCUSSION

The foregoing evidence, derived from three completely different physical methodologies in three independent laboratories, indicates that there is something different about the 0.5 - 2.0 u of bone comprising the wall of the osteocyte and canalicular lumens which contain living cells. By and large this differences disappears with the death of the cell and the necessary inference is that the metabolic activity of the cell is somehow responsible for it. Examples of pathological distortion of this bone wall, the halo volume, are given, suggesting that further study of this cell would be fruitful.

Five specific points merit comment.

1. *Unstable Physical State:* The surprising sensitivity of the permeability and difference in refractive index characteristic of H. V. in perfectly fresh material to procedures in common use brings Dallemagne's work and views to mind.[1] He feels that the in vivo ultrastructure of the bone mineral is not hydroxyapatite but some other state. Conversion to hydroxyapatite occurs after death since this is the stable structure in the absence of the (unknown) living chemical environment. Accordingly he feels that most of the investigations done on bone salt ultrastructure have been done on artifact.

   Our present work has demonstrated some unstable state in H. V., which is permeable in perfectly fresh material but impermeable after numerous treatments which are in common use in tissue laboratories. It must remain for future work to reveal if Dallemagne's structural concepts are valid. It is possible that both he and his critics will prove partially correct.

   The H. V. sensitivity suggests that it would be worthwhile to check such matters as x-ray diffraction patterns on fresh, still moist bone.

2. *H. V. and Parathormone:* In view of the enlarged H. V. in rats after administration of parathormone it seems reasonable to assume that parathormone exerts a definite effect on osteocyte metabolism which in turn causes enlargement of H. V. One further step leads to the suspicion that some of the skeletal calcium load cast out through the urine in hyperparathyroidism arises in H. V. rather than through osteoclastic resorption of bone. Citrate metabolism has often been suggested as the chemical route by means of which this loss might occur.[11,12,13]

3. *Size Effect:* The consistent discrepancy in H. V. permeability between large and small ions suggests a size discriminatory factor operates in normally mineralized bone. The effect is that of reduction in permeability to progressively smaller ions as mineral density progressively approaches its maximum. The causes of this effect other than mineral density and ionic size are not apparent and are probably complex. This effect has been suspected previously[11] and we have now provided direct evidence for its existence. A size discriminatory effect would have important applications to blood-bone exchange processes and the physiology of osteocyte, osteoblast and osteoclast.

4. *H. V. and Blood-Bone Exchange:* The observed failure of the major fraction of a bone ion-sodium for example—to equilibrate with an injected radioactive tracer was a major factor in the development of the defect crystal theory which holds that the nonequilibrating fraction is locked in the crystal lattice or hydration shell.[12] Inherent in this theory is the assumption that the tracer has free access to the crystal if not to its individual atoms.

   More than a dozen anions and cations tested in this laboratory failed to permeate beyond the H. V. in 48 hours.[4] This suggests that the assumed ready

access of all the crystallites to an injected tracer assumes too much, a suspicion others have also entertained.[11]

We suggest that there are other factors which affect blood-bone exchange in addition to the purely passive diffusion of ions among the crystallites and that H. V. is one of them. In effect, H. V. would be a quickly available reservoir of bone permeable to small ions, adjacent to the osteocyte and the diffusion gradients which enable it to live, and thus a more ready donor or acceptor volume of bone in time of need than the remainder of the intercanalicular and interlacunar bone, which in vitro at least is highly impermeable over 48 hour periods.

5. *H. V. and Vitamin D Resistant Rickets:* The large H. V. seen in our case and in others, Engfeldt and coworkers among them, by microradiography, suggests that these peculiar H V. are one of the characteristic pathological facets of the disease. They also add weight to Frost's suggestion that there is a chemical disturbance in osteocyte metabolism in this disease which might have a common chemical denominator with that in the renal tubule. Since the disease is genetically transmitted this is all the more reasonable.

## CONCLUSION

The preceding thoughts will, it is hoped, prove stimulating. The existence of H. V. should allow us to turn the tables on the osteocyte. Previously it has remained hidden in its bony shell from our investigative weapons. Now an alteration in H. V. size or pattern should permit us, by using the alteration as an indication of some disturbance in cell metabolism, to turn the obscuring shell against the cell and make it yield useful information.

## SUMMARY

Evidence is presented indicating that 0.5 - 2.0 u of the bone wall of the lacunar and canalicular lumens which contain live osteocytes are quasipermeable in the normal, perfectly fresh state. Numerous procedures which are routine in histopathology remove this permeability. Disturbed halo volumes—the designation of the permeable shell by the authors—have been found by the present and previous workers in rats given excessive parathormone and in human vitamin D resistant rickets.

The study of H. V. alterations is expected to implement study of osteocyte metabolism.

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