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DEPENDENCE OF MEDULLARY HEMATOPOIESIS ON CLOSE CONTACT WITH AN ABUNDANCE OF HEALTHY CELLS OF THE ENDOSTEUM *

By

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ABSTRACT

This report presents a series of experiments related by the fact that each involves both marrow and its surroundings of endosteum and bone. Previous studies have shown a positive correlation between hematopoietic marrow and magnitude of blood flow to immediately adjacent bone. The studies presented here confirm that relationship and establish a further correlation between distribution of abundant endosteum and hematopoietic marrow. Thus, a rich red marrow, a rich endosteal layer, and a rich bone blood supply, not surprisingly, go together. The general conclusion is that cells of the endosteum may play a vital role in hematopoietic marrow proliferation.

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INTRODUCTION

The relationship between bone and hematopoietic marrow would be self-evident but for the fact that many bones of the normal adult skeleton and, more important, parts of a given bone, may be filled with fat rather than hematopoietic marrow. That inconsistency plus the fact that extramedullary hematopoiesis occurs in health and disease has directed attention away from bone as playing a major role in supporting hematopoiesis. The fact that the proximal end of the normal femur may contain rich red marrow while the distal end contains only fat can be interpreted as proof that bone itself is not an important factor in hematopoiesis or that the bone at the proximal end is functionally quite different from the bone at the distal end.

In spite of 100 years of intense investigation of marrow physiology and pathology since Neumann's discovery (1868) that blood cells develop in the red bone marrow (1), little light has been shed on the possible importance of the surrounding bone in the process. The fact that under normal and pathological circumstances hematopoiesis may occur in extramedullary sites probably accounts for the fact that bone has not been attributed great importance in the maintenance of marrow function. On the other hand, in most adult mammals it is within a surrounding of bone that marrow thrives. The question as to what a surrounding of bone may contribute to the welfare of its contained marrow has been the subject of recent investigations in this and other laboratories (2, 3). Rohlich's original studies on this question (4) led him to conclude that the bone was important in modifying the blood supply of the medullary cavity and

that regeneration of marrow following mechanical removal occurred in tissue which apparently had its origin in the Haversian canals.

It is the object of these studies to demonstrate through a series of experimental results that the distribution of hematopoietic marrow in the skeleton corresponds to the distribution of large numbers of "healthy" endosteal cells and to suggest that an abundance of healthy endosteal cells generate, within their immediate environment, the inducer for circulating primitive stem cells to become committed hematopoietic stem cells.

MATERIALS AND METHODS

Hypertrophy of endosteum after destruction of marrow by mild radiation: Adult female mice of the LAF_1 strain were irradiated with 1100 rad ^{60}Co for this experiment, following the protocol set forth by Till and McCulloch (5) for spleen colony studies. Ten days after irradiation the mice were killed and the femur fixed in Bouin's for sectioning.

Mechanical removal of marrow: Rats and mice were subjected to "endosteal curettage" of the left femur, entering from the distal end with a #54 twist drill for rats and a #73 drill for mice. This size drill just fit the cavity of the shaft, completely disrupting and removing marrow as well as curetting the inner surface of the bone. At the proximal and distal ends some hematopoietic marrow remained intact. On various days after curettage 10 μCi ^{59}Fe were injected intravenously via tail vein. Four hours later the animals were decapitated, both femurs were removed, cleaned and cut into proximal, middle, and distal segments. The central section was fixed in Bouin's and counted in a well-type scintillation counter for determination of ^{59}Fe content. The results were expressed as percent

in the operated femur of ^{59}Fe content in the unoperated femur.

On various days after endosteal curettage rats were killed to determine the extent of regeneration. The mid-femoral shaft segments were decalcified in Decal, imbedded in paraffin, sectioned at 6 μ, and stained with alum hematoxylin and eosin.

Distribution of marrow and bone cells in a normal monkey: In order to map the distribution of erythropoietic marrow, a normal adult cynomolgous monkey was given 50 μCi ^{52}Fe and 18 hours later (when most of the iron had been incorporated into hemoglobin in the marrow) the animal was perfused with saline followed by formalin. Positron scintillation camera pictures (6) were taken of the central skeleton (with extremities, spleen and liver removed) and of the extremities (clavicle, scapula, humerus, and femur), liver and spleen. From the map of the distribution of hematopoietic marrow, areas of the skeleton representing active and inactive marrow were chosen for histologic study.

Four young monkeys shown by ^{52}Fe studies to have erythropoietic marrow in their tibias were given 2000 rad x-rays to one tibia, and in two of them that tibia was subjected to endosteal curettage 3 months later. There was complete absence of ^{52}Fe uptake in the irradiated tibias prior to curettage and failure of erythropoietic marrow regeneration following curettage. The bone hyperemia (increased ^{18}F uptake--7) which characteristically follows curettage of the monkey tibia failed to occur in the radiated portion of the bone (2). Sections of the radiated tibias were examined for bone and marrow histology.

Effect of hypophysectomy, growth hormone, and ACTH on marrow regeneration: Female rats of the Long-Evans strain were hypophysectomized at 28 days of age. Three days later these and normal controls of the same age were subjected to endosteal curettage of the left femur. Immediately after curettage the rats were divided into 3 groups, each group having the same average body weight. One group of 6 rats received 1/2 unit growth hormone (porcine-grade B) intraperitoneally daily for 6 days, after which the dose was increased to 1-1/2 units daily until sacrifice. A second group of 13 rats received 4 units ACTH (Armour H.P. Acthar Gel) subcutaneously daily until sacrifice. A third group of 14 hypophysectomized rats served as uninjected controls. Another group of hypophysectomized rats were bled 1/3 of the blood volume by cardiac puncture on the day of curettage and on the following day. During the next 2 weeks they were bled 1/3 of blood volume at various intervals to maintain a hematocrit around 25%.

Completeness of hypophysectomy was determined by body weight gain during life and at autopsy by examination of the pituitary site using a dissecting binocular microscope.

RESULTS

Hypertrophy of endosteum after destruction of marrow by mild radiation (1100 rad): Female mice of the LAF_1 strain receiving 1100 rad ^{60}Co were used in this part of the study 10 days post-radiation. The difference in radiosensitivity of bone and marrow is such that a radiation dose sufficient to cause complete necrosis of marrow (1100 rad) in the mouse is not associated with loss of osteocytes or endosteal cells but rather a

significant degree of hypertrophy of the endosteum. While all marrow elements are undergoing rapid and complete necrosis, the endosteal cells become larger and more abundant, Fig. 1.

Marrow regeneration following mechanical removal: In the study of regeneration of marrow following mechanical removal from the femoral shaft of the mouse, it was found that regeneration in LAF_1 mice was surprisingly slow considering the small diameter of the wound and the proliferative capacity of the marrow, complete regeneration requiring 40 to 50 days, Fig. 2. Histologic examination showed that mechanical disruption of the marrow resulted in loss of endosteum and loss of stainable osteocyte nuclei from most lacunae of the inner surface of the bone, Fig. 3. Regeneration of the marrow occurred only after new bone replaced the apparently infarcted inner half of the femoral shaft or after newly formed bone spicules with hypertrophied endosteum appeared in the medullary space. Variations in rate and pattern of recovery of marrow within the cavity of LAF_1 mice could be correlated with the number of stainable endosteal nuclei in the immediate vicinity of the marrow at all postoperative intervals, Figs. 4 and 5.

Because of histologic evidence that in this strain of mice curettage results in infarction of the inner 1/2 of the bone in the region of the femoral shaft, the amount of blood contained in the bone was studied at various intervals following endosteal curettage. Radioiodinated albumin (RISA) was injected intravenously and 6 minutes later the curetted

and contralateral normal femur were removed. The radioactive content of the mid-shaft segment of each bone was determined with the medullary contents intact and after removal. The results are shown in Fig. 6. As can be seen from the figure, the amount of RISA in the bone was 1/2 that in the normal femoral shaft 4 days after curettage but rose to more than twice normal by 15 days. This result is similar in timing and magnitude to that previously found after endosteal curettage in rabbits using ^{18}F as an indicator of bone blood flow (7). Note from the figure that the post-operative blood clot had been vascularized to the extent of containing a normal amount of the injected RISA by 11 days post-curettage.

Distribution of endosteal cells and hematopoietic marrow in a normal monkey: On the basis of observations from abnormal situations, it was decided to investigate distribution of endosteal cells and hematopoietic marrow in the healthy animal.

The distribution of erythropoietic marrow in a normal adult monkey was mapped by the use of ^{52}Fe and the positron scintillation camera (6), Fig. 7. As can be seen from the figure, the proximal end of the femur contained erythropoietically active marrow, which terminated abruptly at the mid-femur, the distal end being completely inactive. Sections were made from the active (proximal) and inactive (distal) ends of the humerus and femur. In the healthy monkey there was no apparent difference in the number or morphology of the osteocytes in the active and inactive areas. However, a much more cellular endosteum was found adjacent to areas of active marrow than adjacent to fatty marrow.

When, in the monkey, an hematopoietically active bone was given 2000 rad, hematopoiesis was permanently abolished and there was failure of marrow regeneration following mechanical removal and endosteal curettage (2). Microscopic examination revealed absence of osteocytes, no endosteal cells, and a marrow cavity filled with fibrous tissue. Endosteal curettage done 3 months post-radiation did not stimulate significant new bone formation or regeneration of hematopoietic or fatty marrow, but resulted in a primarily fibrous replacement, Fig. 8.

Effect of hypophysectomy, growth hormone and ACTH on marrow regeneration: Because of foregoing evidence that healthy bone and endosteum are prerequisites to a healthy hematopoietic marrow, it was decided to investigate the effects on marrow regeneration of hormones with known influence on bone cells. The effect of hypophysectomy (with growth hormone or ACTH replacement) was investigated.

The extent of regeneration of hematopoietic marrow in the mid-shaft of curetted femurs was demonstrated by comparison of the radioactivity of the curetted femur to that of the contralateral, unoperated femur 4 hours after intravenous injection of ^{59}Fe, Fig. 9. Regeneration was also estimated by microscopic examination. As can be seen from Fig. 9, in normal rats complete regeneration of hematopoietic marrow required approximately 20 days. Hypophysectomy resulted in a significant delay, complete recovery requiring 40 days. Severe bleeding had no effect on rate of regeneration. The administration of growth hormone to hypophysectomized

rats resulted in a rate of recovery comparable to normal, i.e., 20 days, Figs. 9 and 10. Administration of ACTH resulted in permanent arrest of the recovery process at the stage of trabecular bone formation, Figs. 9 and 11.

Hypertrophy of endosteum after destruction of marrow by radiation: The difference in radiosensitivity of bone and marrow is such that a radiation dose sufficient to cause rapid, complete necrosis of marrow in the mouse (1100 rad) is not associated with loss of osteocytes or endosteum, but on the contrary, endosteal cells become larger and more abundant, Fig. 1. At this radiation dose no spontaneous recovery of marrow occurs, indicating that none of the remaining cells (endosteum, osteocytes, cells in the Haversian canals, etc.) has stem cell competence. However, since this is the standard preparation for marrow transplant studies, it is apparent that exogenous stem cells thrive in this environment of hypertrophied endosteum. Within 10 days after an infusion of compatible stem cells, the medullary cavity may be filled with "colonies", in which case the endosteum will have been maintained in its usual state (a single thin layer). From the point of view of this study it is tempting to propose teleologically that the endosteum hypertrophies in an attempt to stimulate the mortally injured medullary elements. When a competent exogenous stem cell is introduced into the bloodstream of such an animal, it survives and flourishes in a surrounding of endosteum (medullary cavity) or in the spleen. It is suggested that contact with large numbers of healthy endosteal cells (or similar cells in the spleen) may be necessary for stem cell growth, i.e., that a primitive cell is induced into hematopoietic

activity by information received from endosteal cells. Doses of radiation large enough to produce permanent loss of osteocytes and endosteal cells result in permanent marrow aplasia, Fig. 8.

Marrow regeneration following mechanical removal: In normal rabbits (2) and rats (9) hematopoietic marrow regenerates after mechanical removal. After removal of the marrow the clotted blood which fills that cavity undergoes a series of changes reminiscent of embryologic marrow development. Whether the factors responsible for this rapid and complete conversion of a blood clot to normal, functioning marrow are local or systemic has not been clarified but may represent elements of both.

In this study variations in rate and pattern of recovery of marrow in the femoral shafts of rats and mice could be correlated with the number of stainable endosteal nuclei in the immediate vicinity of the newly regenerated marrow at all post-operative intervals and experimental conditions, Figs. 4 and 5. Loss of endosteum and osteocytes from the inner portion of the bone following mechanical removal appeared to determine rate of hematopoietic recovery. Preserved or regenerated endosteal cells, either in non-infarcted or re-formed shaft or in newly formed trabeculae within the cavity were apparently needed before marrow regeneration occurred.

In previous studies on rabbits, in which regeneration of marrow after mechanical disruption was shown to be relatively slow, studies of bone blood flow using ^{18}F uptake showed evidence of infarction of the operated bone (2). This result was subsequently confirmed by studies of appearance of a labeled plasma protein (blood content) in the bone substance of the operated and unoperated contralateral femur one week

after operation, Fig. 12. As can be seen from the figure, the mid-shaft segment of the bone remained essentially avascular (completely infarcted) 7 days after mechanical disruption of the marrow. This massive infarction of the shaft following removal and immediate replacement of the marrow in rabbits is presumably responsible for the slow recovery of hematopoietic marrow previously shown to occur in such animals (2).

Distribution of endosteal cells and hematopoietic marrow in a normal monkey and in mice: Mice, rats and rabbits have hematopoietic marrow widely distributed throughout the skeleton, but old dogs and monkeys, like adult man, have regional distribution of hematopoietic marrow. After mapping the distribution of erythropoietic marrow in an adult monkey, sections were taken from active (proximal) and inactive (distal) parts of humerus and femur. Microscopic examination revealed no apparent difference in osteocytes, but a rich endosteal layer was found only in the areas of active hematopoiesis.

In the monkey, blood flow to the skeleton has been shown (with some exceptions) to have the same distribution as erythropoietic marrow (10). The distribution of skeletal blood flow, abundant endosteum and hematopoietic marrow are sufficiently similar to suggest the possibility of interdependence. Mechanical disruption of marrow results in marked endosteal proliferation (9), marked increase in bone blood flow (2), and stimulation of hematopoietic marrow (2). These findings imply that a healthy marrow depends on a healthy endosteum and a good blood supply.

In normal rats and mice hematopoietic marrow extends throughout the length of humerus and femur but is entirely absent from all but the most

proximal of the caudal vertebrae (11). In areas of active hematopoiesis a continuous layer of endosteal cells is readily apparent. In caudal vertebrae from the mid-portion of the tail of mice where the medullary cavity is filled with fat, a good endosteal layer is seen in many areas of the vertebral body without associated hematopoietic marrow. Thus the association between morphologically intact endosteum and adjacent hematopoiesis, like the association between bone blood flow and hematopoiesis, is not without exceptions. Although the distal caudal vertebrae of the mouse have an intact endosteum, the bone blood supply is small (compared to hematopoietically active areas). Again one may conclude that hematopoietic marrow requires abundant endosteum associated with an active bone blood flow to flourish.

Effect of hypophysectomy, growth hormone and ACTH on marrow regeneration: The finding that hypophysectomy significantly delayed regeneration of marrow after mechanical removal was not surprising, since the metabolic rate is reduced to half normal. Since new bone formation is a prominent step in marrow regeneration, growth hormone was given with the expectation that it might intensify bone formation and delay the regeneration process. It was quite unpredicted that administration of growth hormone would result in a marked reduction in duration of the osseous phase of recovery with rapid erosion of the newly formed bone and replacement by hematopoietic marrow. Growth hormone administration completely nullified the effect of hypophysectomy, Figs. 9 and 10. Administration of growth hormone (1-1/2 units per day) to normal LAF_1 mice with a slow marrow regeneration rate (Fig. 2) was without effect, indicating that growth hormone is not a

specific stimulator of hematopoietic elements but is necessary for normal bone repair which is a prerequisite to hematopoietic regeneration.

Replacement therapy with growth hormone resulted in re-establishing the normal pattern of rapid erosion and replacement of the infarcted inner portion of the shaft and trabecular bone formation and resorption in the cavity. In other words, it permitted repair of the wound and re-establishment of normal bone structure and function. As in the normal animal, this repair process is associated with marked endosteal proliferation, followed by osteoclastic endosteum and by 15 days, re-establishment of a normal appearing endosteum. As in the normal rat, islands of regenerating marrow appeared in a surrounding of hypertrophied endosteum.

Equally surprising was the finding that administration of ACTH to hypophysectomized rats resulted in permanent arrest of the repair process at the stage of trabecular bone formation, Figs. 9 and 11. Rats in this group (hypophysectomized, marrow mechanically removed and ACTH injected) never entered the stage of trabecular bone resorption and hematopoietic proliferation even if ACTH administration were discontinued (ACTH for 68 days, autopsied 24 days after ACTH stopped; ACTH for 18 days, autopsied 9 days later). This experimental combination produced localized permanent myelofibrosis, Fig. 11, comparable to that seen in severe myelofibrosis in human beings, Fig. 13. Large portions of dead (infarcted) cortical bone remained, as evidenced by vacant lacunae, and the newly formed trabecular bone had a discontinuous and sparse endosteum characteristic of that seen in inactive areas of the normal monkey and in the trabecular bone from patients with myelofibrosis.

The possibility that a growth hormone deficiency is a contributing factor in human myelofibrosis seems to be ruled out by the finding of normal serum growth hormone concentrations in such patients (12). The possibility that the effects of hypophysectomy, growth hormone and ACTH on marrow regeneration were indirect, through their opposite actions on the thymus, was ruled out by finding entirely normal marrow regeneration when thymectomy was substituted for hypophysectomy.

SUMMARY AND CONCLUSIONS

This report presents a series of experiments related by the fact that each involves both marrow and its surroundings of endosteum and bone. Previous studies have shown a positive correlation between hematopoietic marrow and magnitude of blood flow to immediately adjacent bone (10). The studies presented here confirm that relationship and establish a further correlation between distribution of abundant endosteum and hematopoietic marrow. Thus, a rich red marrow, a rich endosteal layer, and a rich bone blood supply, not surprisingly go together. The results of the various experiments presented are as follows:

1. Hypertrophy of the endosteum following radiation destruction of the marrow may result from an attempt to stimulate the mortally injured hematopoietic elements.

2. Contact with large numbers of healthy endosteal cells may be necessary for stem cell growth, i.e., primitive cells may be induced into hematopoietic activity by information received from endosteal cells.

3. Variations in rate and pattern of recovery of marrow after mechanical removal could be correlated with the number of stainable endosteal nuclei in the immediate vicinity of the newly regenerated marrow at all post-operative intervals and all experimental conditions.

4. In a normal monkey a rich endosteal cell layer was found in areas of active hematopoiesis but not in areas of fatty marrow.

5. Hypophysectomy delays marrow regeneration following surgical removal. Growth hormone replacement therapy restores the pattern to normal, whereas ACTH administration inhibits regeneration.

The general conclusion is that cells of the endosteum may play a vital role in hematopoietic marrow proliferation.

DESCRIPTION OF FIGURES

Fig. 1. Comparison of the endosteum covering trabecular bone from the distal end of the femur of a normal mouse (top) and a mouse 10 days after 1100 rad ^{60}Co (bottom). Note the thin single layer of endosteum in the normal mouse as compared to the multi-layer of larger cells adjacent to the bone 10 days after radiation. Magnification: x 472.

Fig. 2. Regeneration of marrow in LAF_1 mice following mechanical removal of marrow from the femoral shaft.

Fig. 3. Appearance of bone and medullary contents in mouse femur 4 days after mechanical removal of marrow. Lacunae adjacent to endosteal surface are devoid of nuclei, indicating infarction of inner surface of bone. The marrow cavity is filled with clotted blood undergoing early organization. Magnification: x 600.

Fig. 4. Appearance of bone and marrow in mouse femur 38 days following mechanical removal of marrow. Most of the lacunae now contain stainable nuclei. The endosteum has regenerated and a mixture of fatty and hematopoietic marrow occupies the adjacent cavity. Magnification: x 600.

Fig. 5. Segment of mouse femur 66 days after mechanical removal of marrow. Throughout most of the bone the osteocytes, endosteum and marrow had healed, but in some areas lacunae remained vacant, new bone had not formed on the endosteal surface, and the adjacent marrow was fibrotic. Magnification: x 600.

Fig. 6. $R^{131}ISA$ content of mid-shaft segment of curetted femur of LAF_1 mice at various times after endosteal curettage, expressed as % of contralateral (unoperated) femur.

DESCRIPTION OF FIGURES

Fig. 7. Positron camera pictures of ^{52}Fe distribution in an adult monkey. Eighteen hours after administration of the isotope the animal was perfused with saline followed by formalin. The viscera and extremities were removed and photographed separately. The figure to the left shows the distribution of erythropoietic marrow in the central skeleton (extremities, spleen and liver removed). The figure to the right shows distribution of marrow in the extremities (clavicle, scapula, humerus and femur), liver and spleen.

Fig. 8. Tibia of monkey 7 months following a dose of radiation sufficient to produce permanent atrophy of marrow (2000 rad X-rays to left leg). Note that lacunae are devoid of osteocytes, there is no endosteal layer, and the marrow cavity is occupied by fibrous tissue. Endosteal curettage done 3 months post-radiation did not stimulate significant new bone formation or regeneration of hematopoietic marrow. Magnification: x 600.

Fig. 9. Regeneration of hematopoietic marrow in the mid-shaft of curetted femur of hypophysectomized rats receiving various treatments as compared to normal rats.

Fig. 10. Appearance of regenerated marrow (17 days after removal) in hypophysectomized rat (left) and hypophysectomized rat given growth hormone starting the day the marrow was removed (right). Magnification: x 39.

Fig. 11. Appearance of the bone and medullary cavity of the mid-shaft of the femur 53 days after hypophysectomy, endosteal curettage, and ACTH administration. The medullary cavity is filled with a syncytium of trabecular bone and loose connective tissue with rare hematopoietic elements. Magnification: x 41

DESCRIPTION OF FIGURES

Fig. 12. Appearance of label in operated and control femur of a rabbit one minute after intravenous injection of ^{59}Fe (7 days post-operative).

Fig. 13. Section through mid-femur of a patient who died with myelofibrosis secondary to polycythemia vera. Note the greatly eroded cortical bone (top half) and abnormal trabecular bone and fibrous tissue replacing normal medullary contents (bottom half). Magnification: x 17.5

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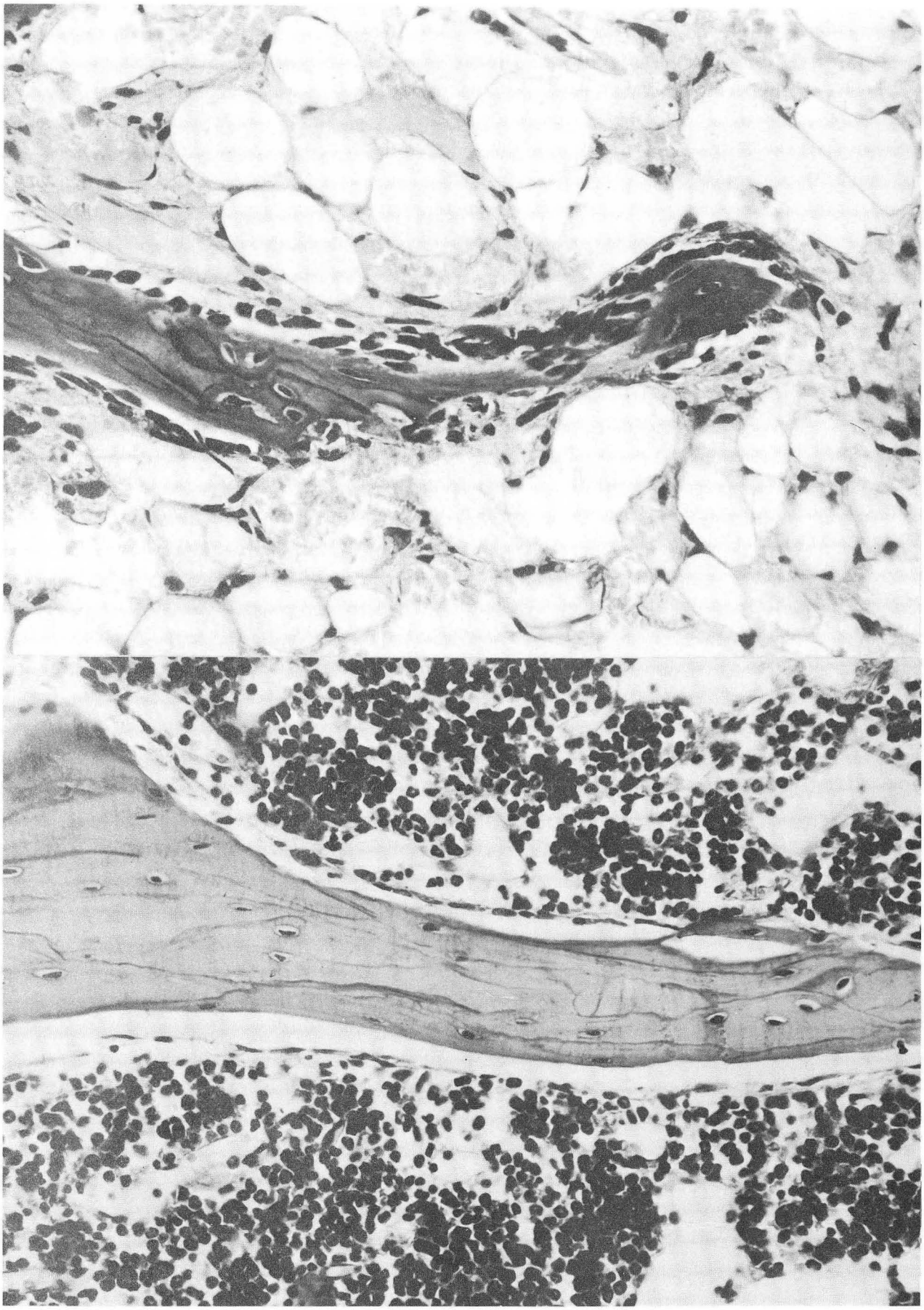

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Fig. 1

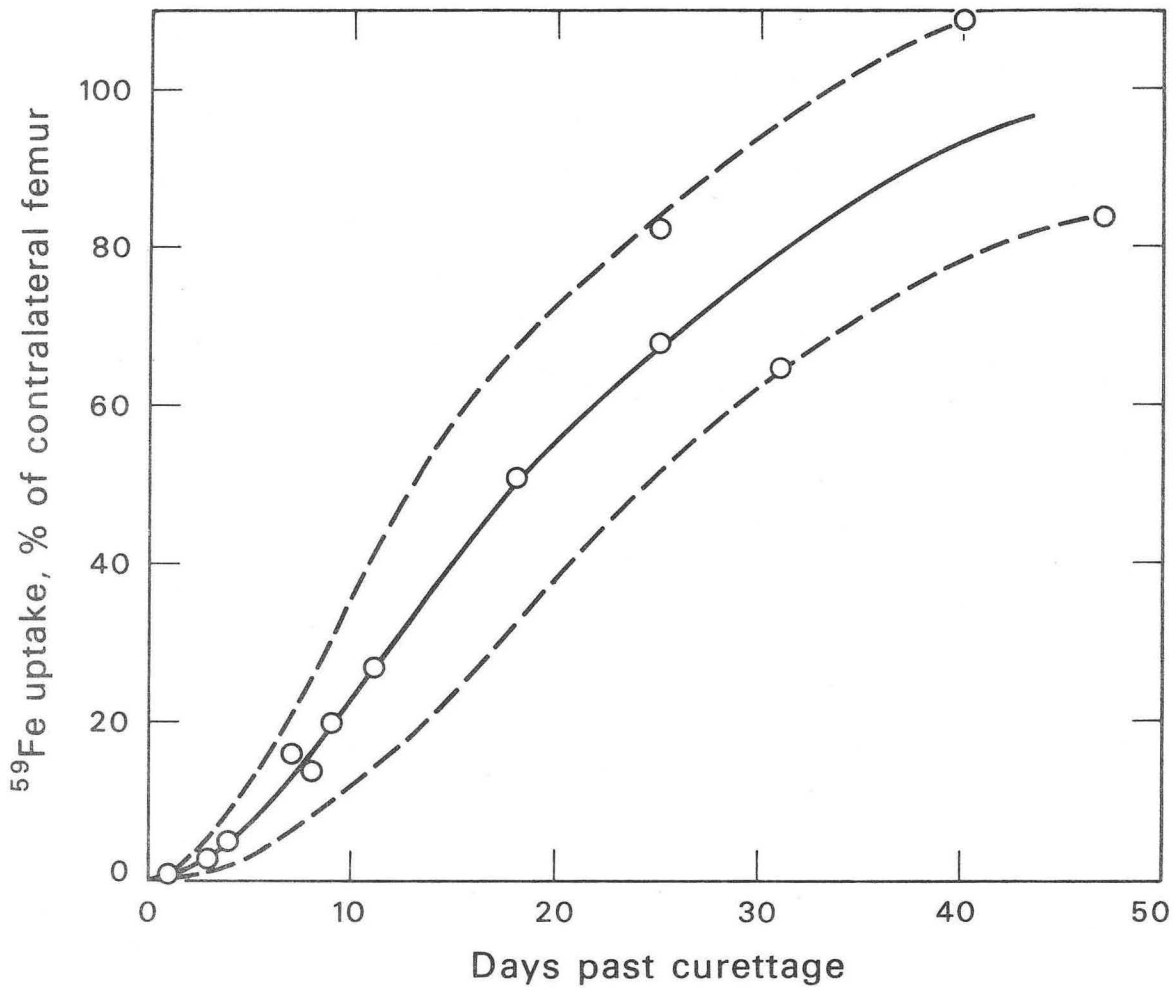

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Fig. 2

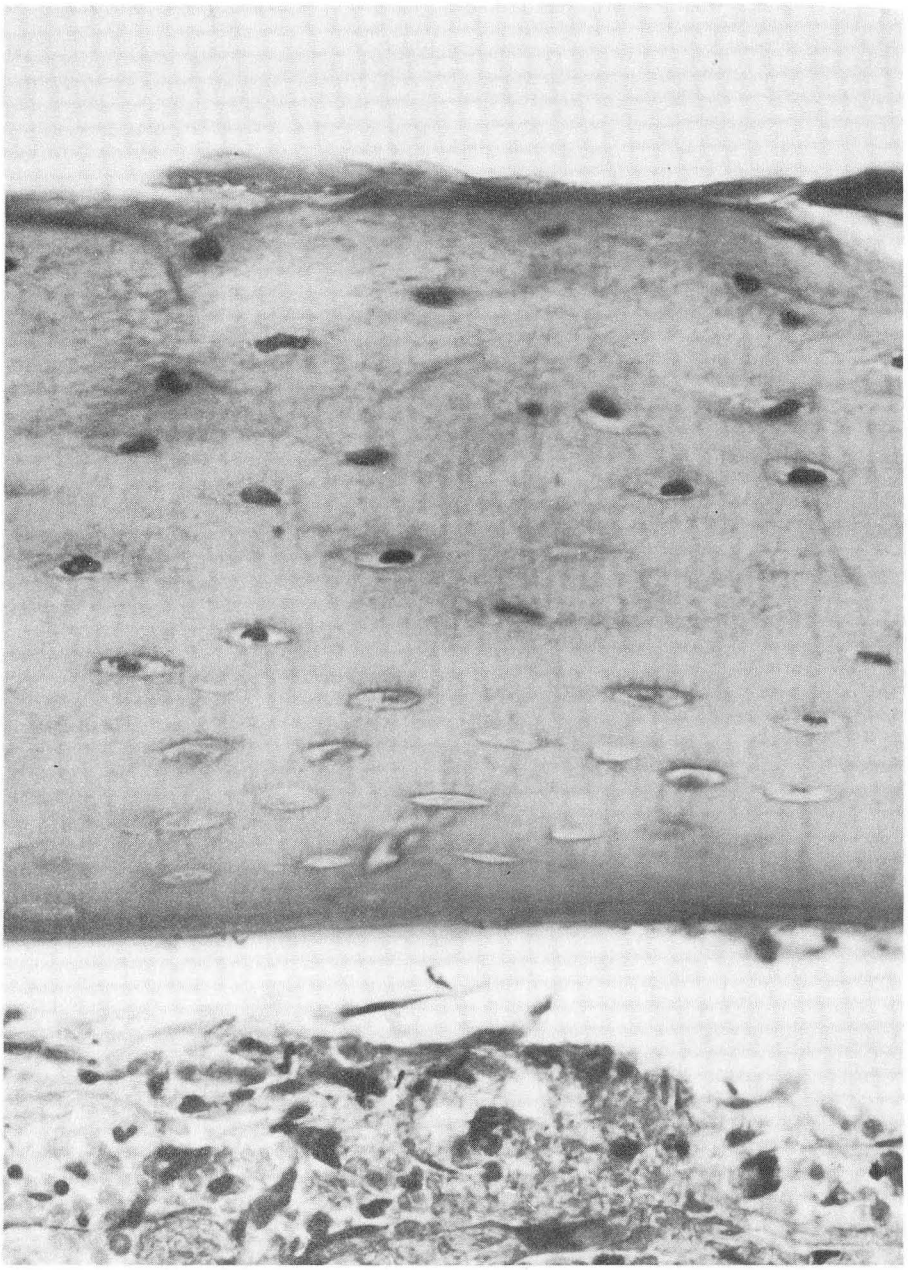

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Fig. 3

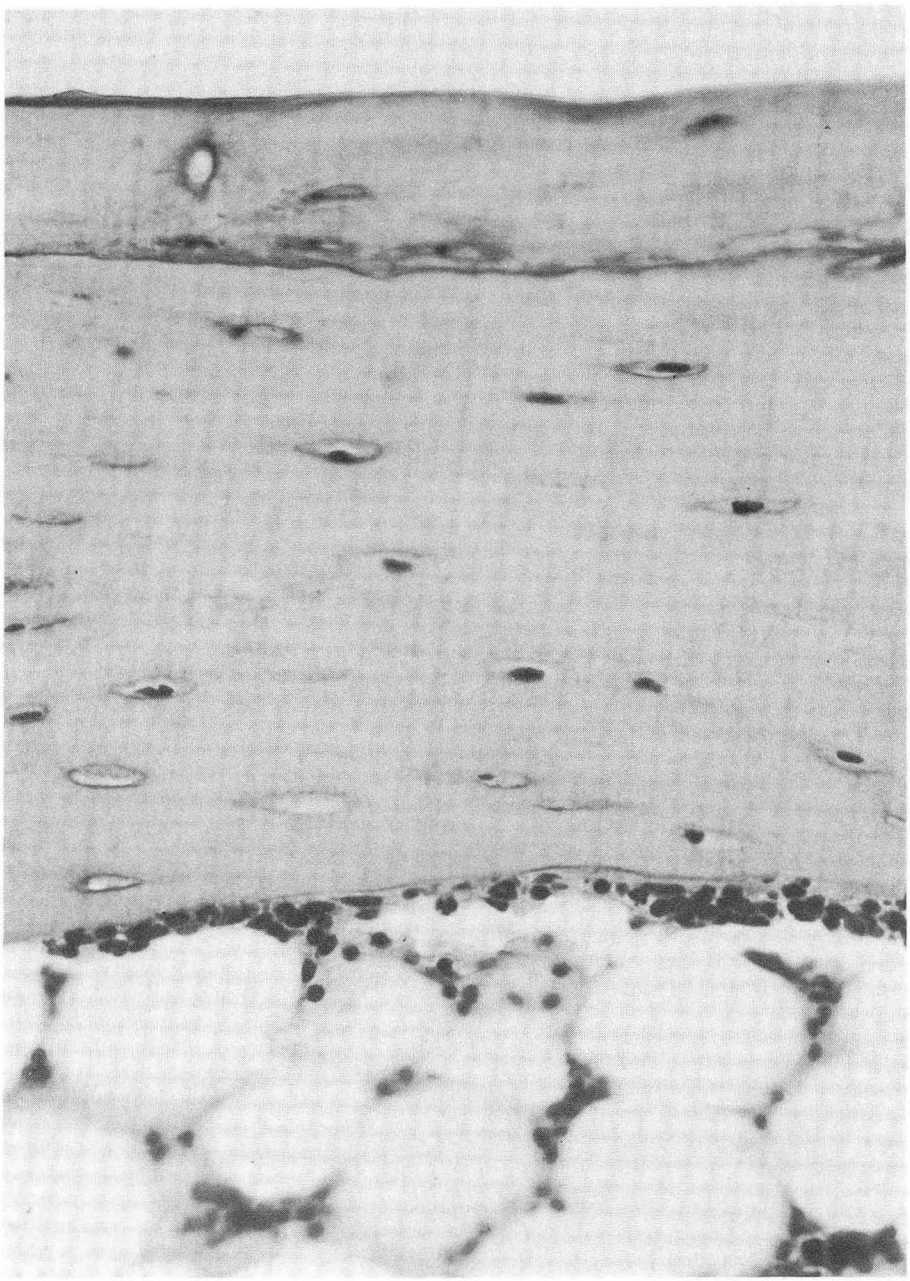

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Fig. 4

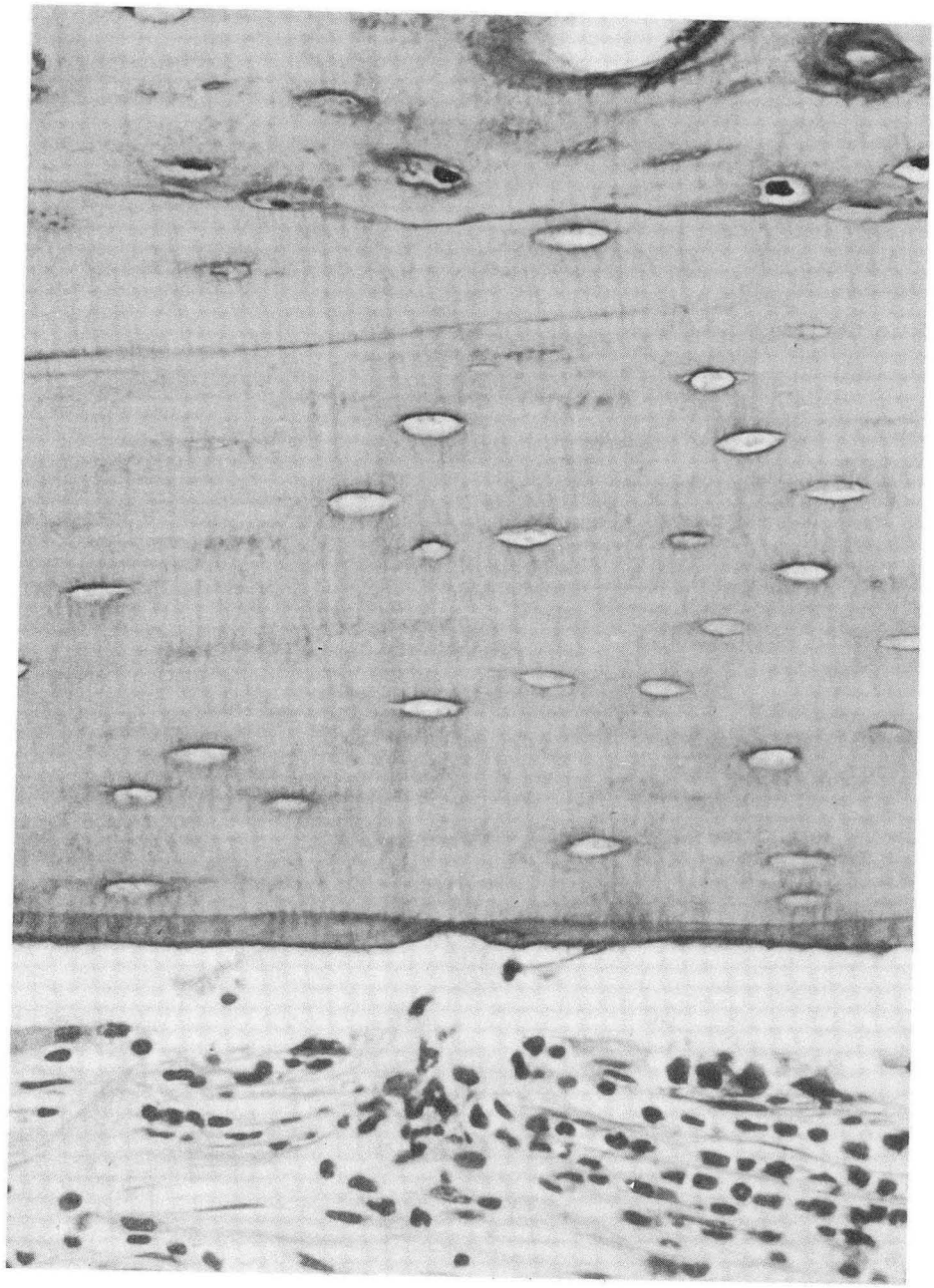

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Fig. 5

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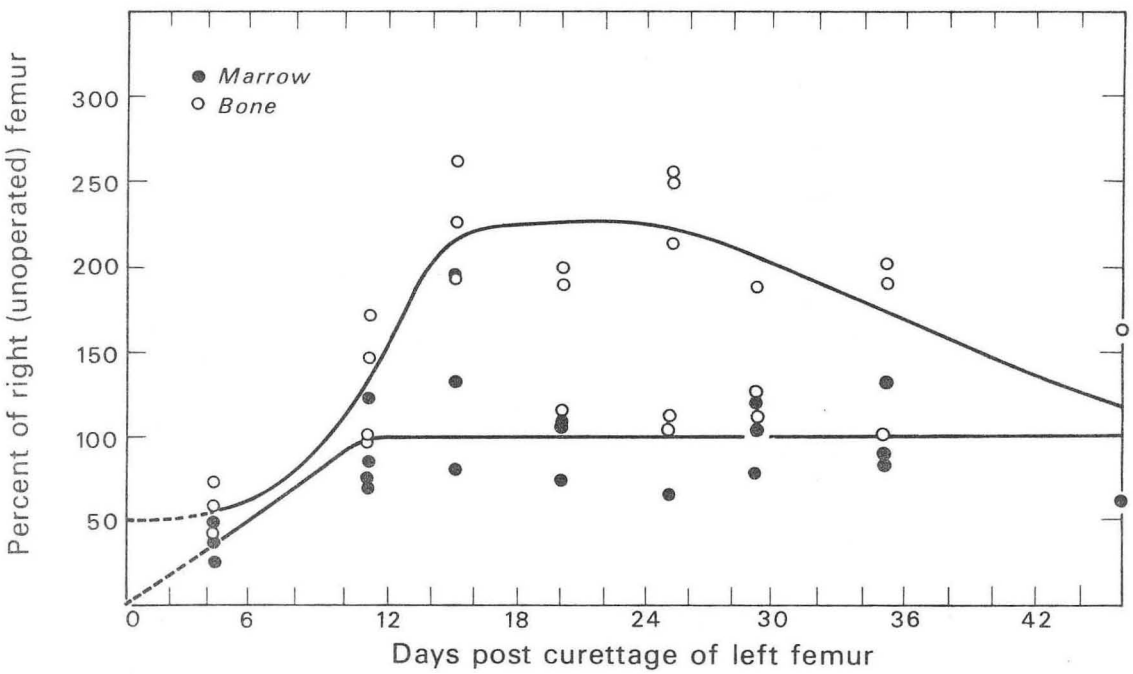

Fig. 6

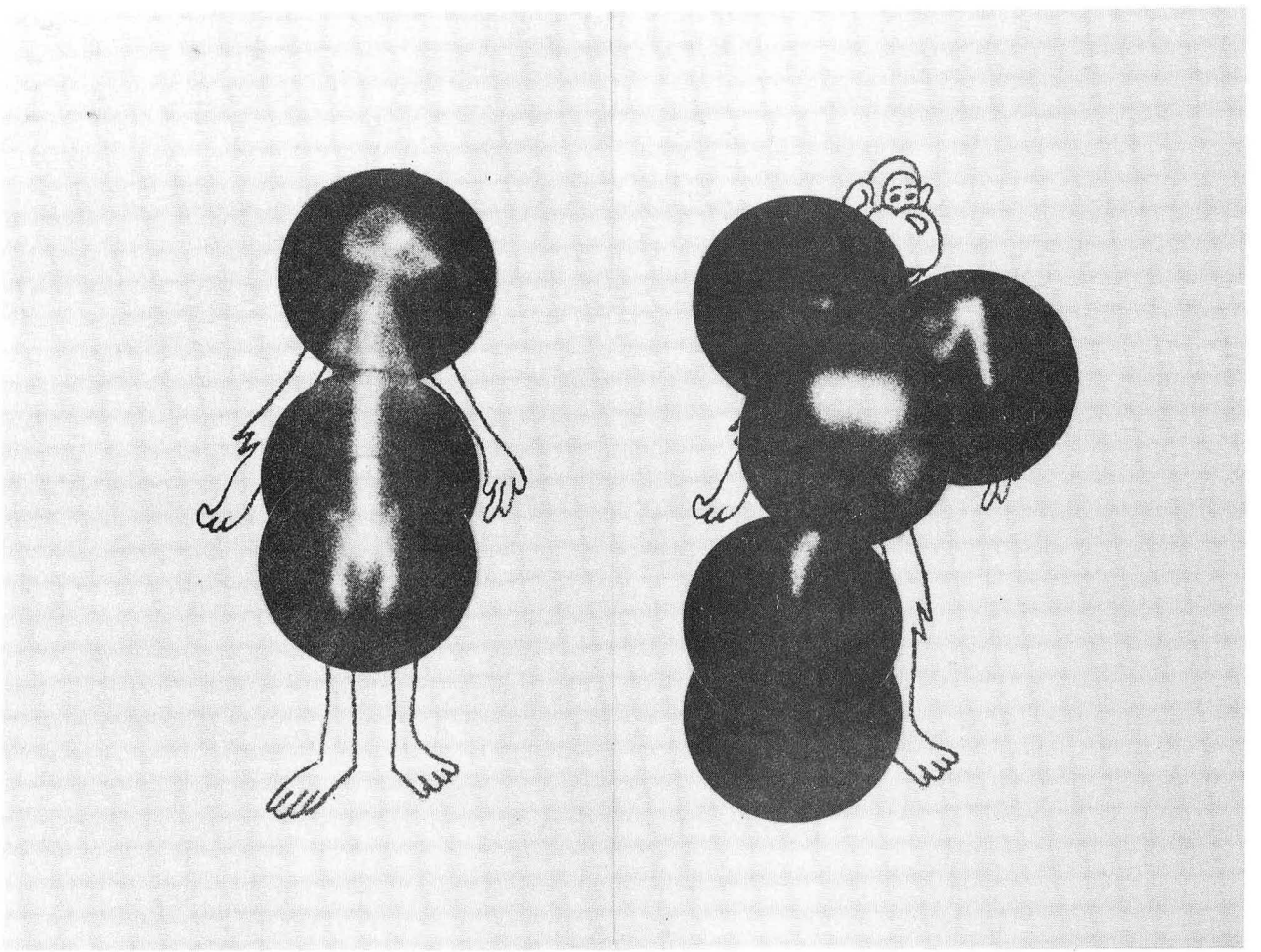

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Fig. 7

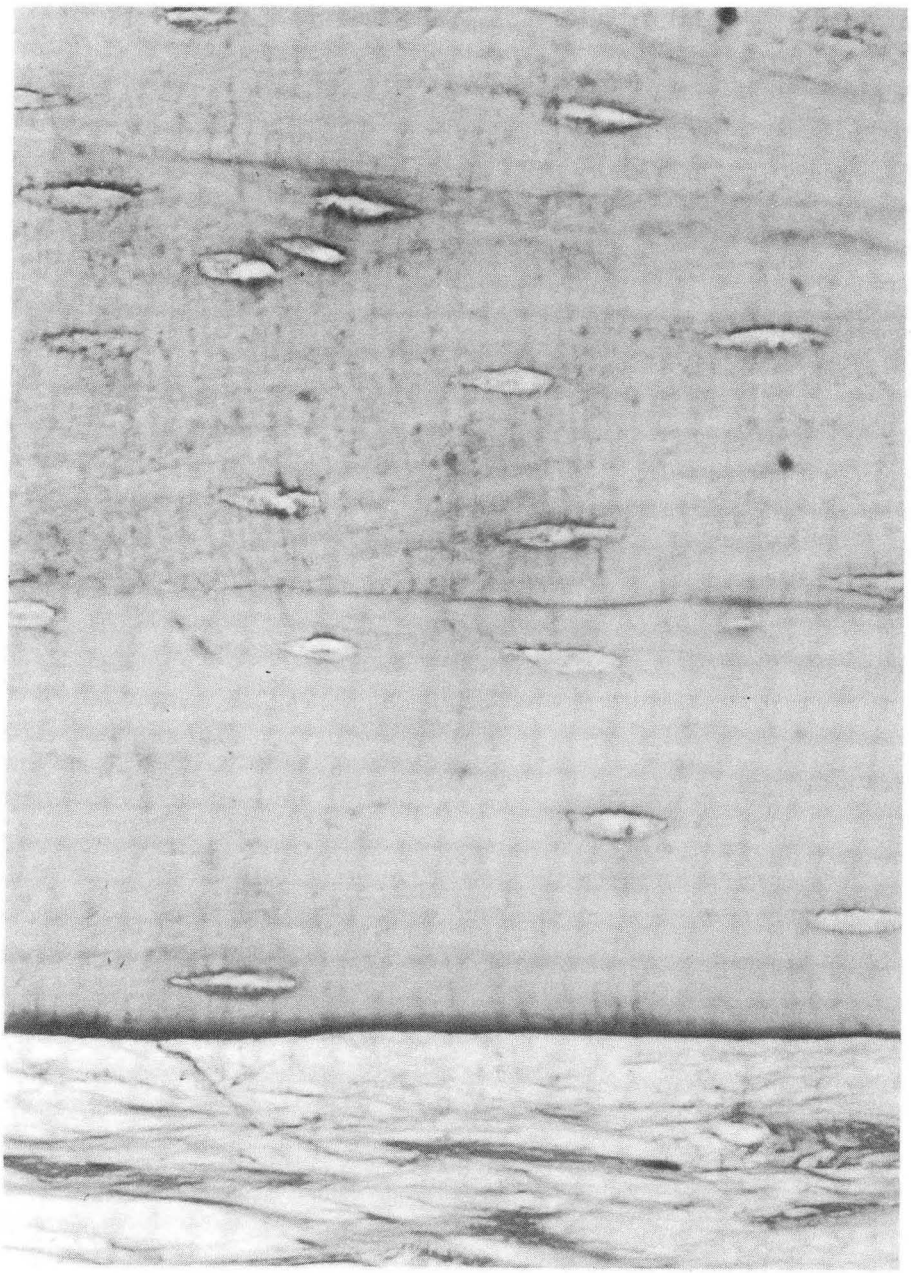

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Fig. 8

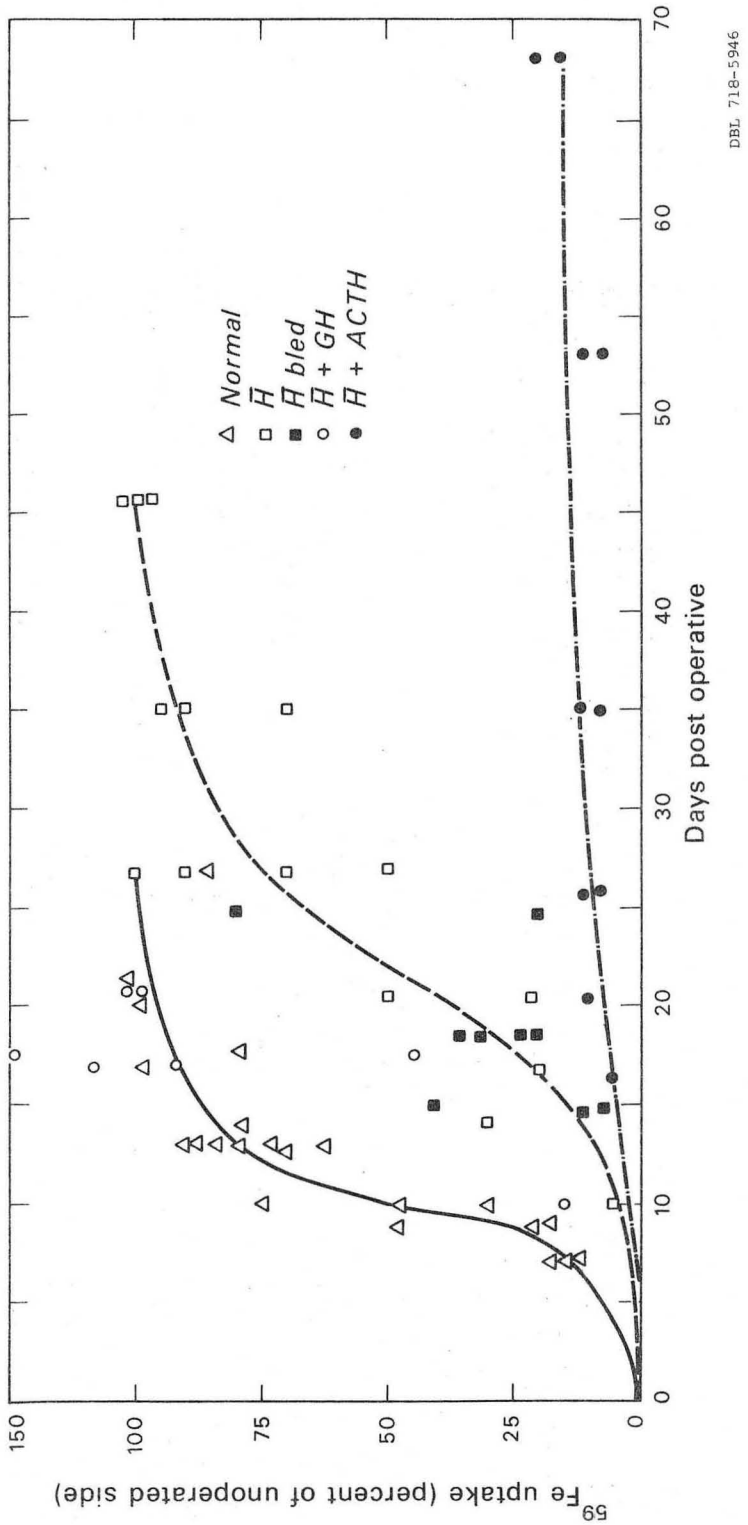

Fig. 9

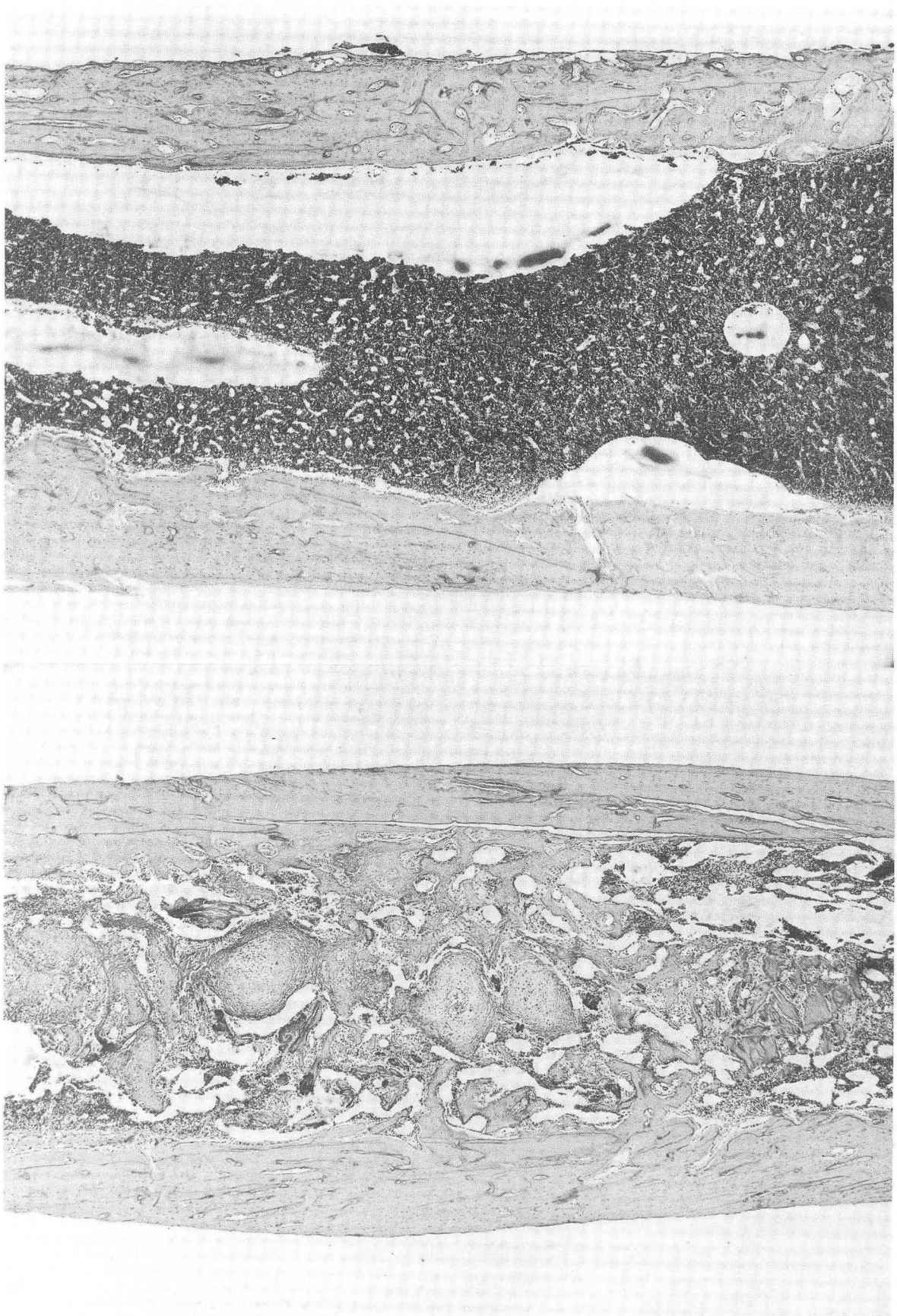

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Fig. 10

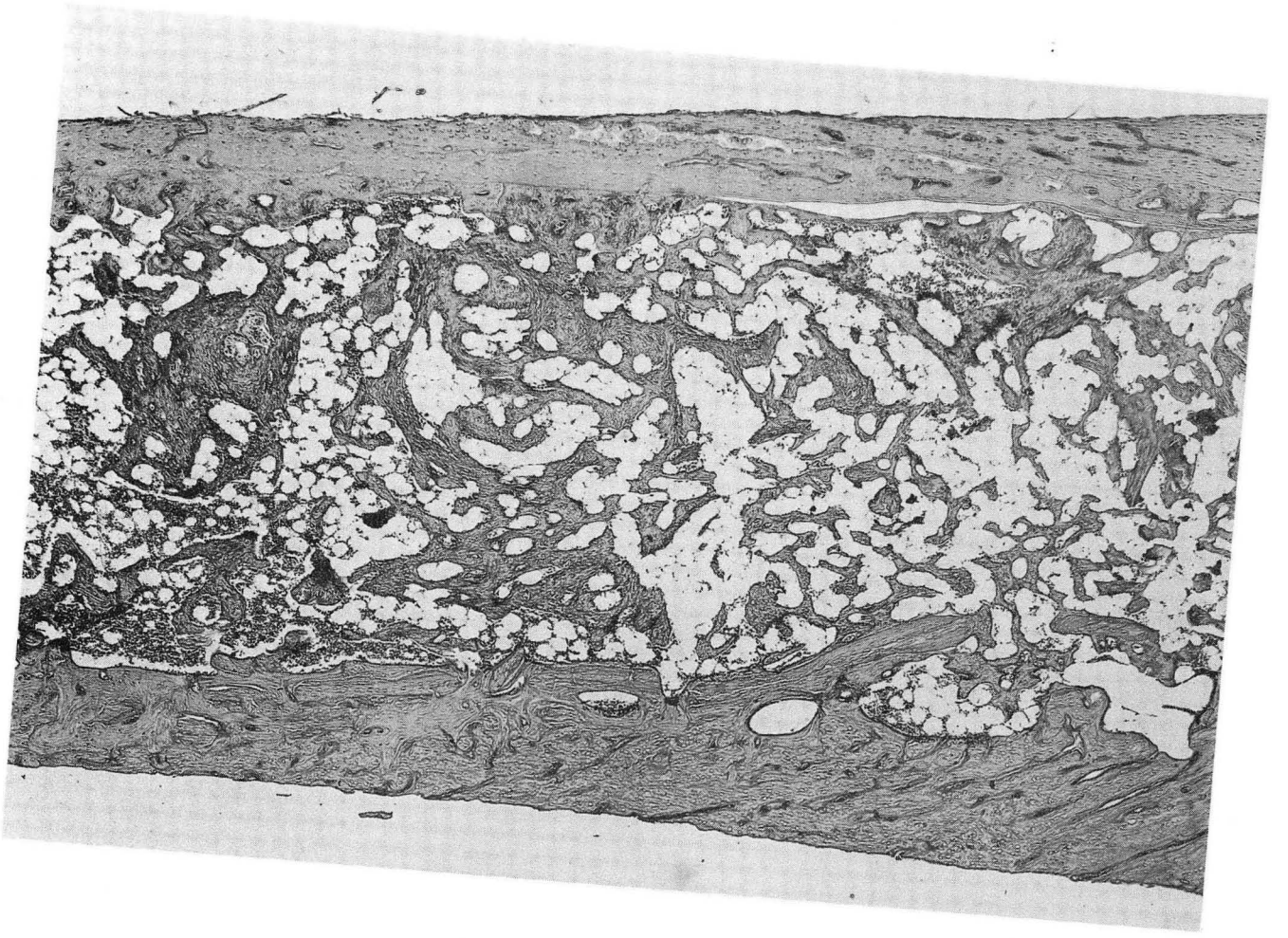

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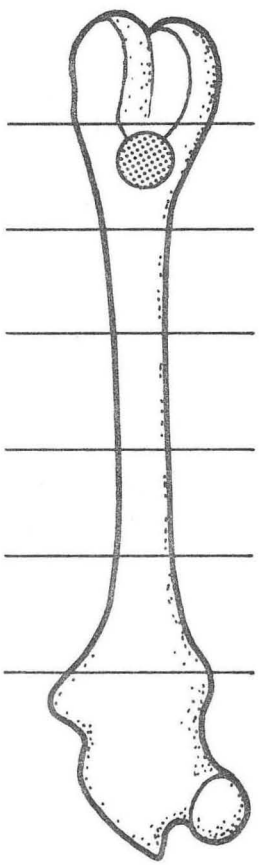
Fig. 11

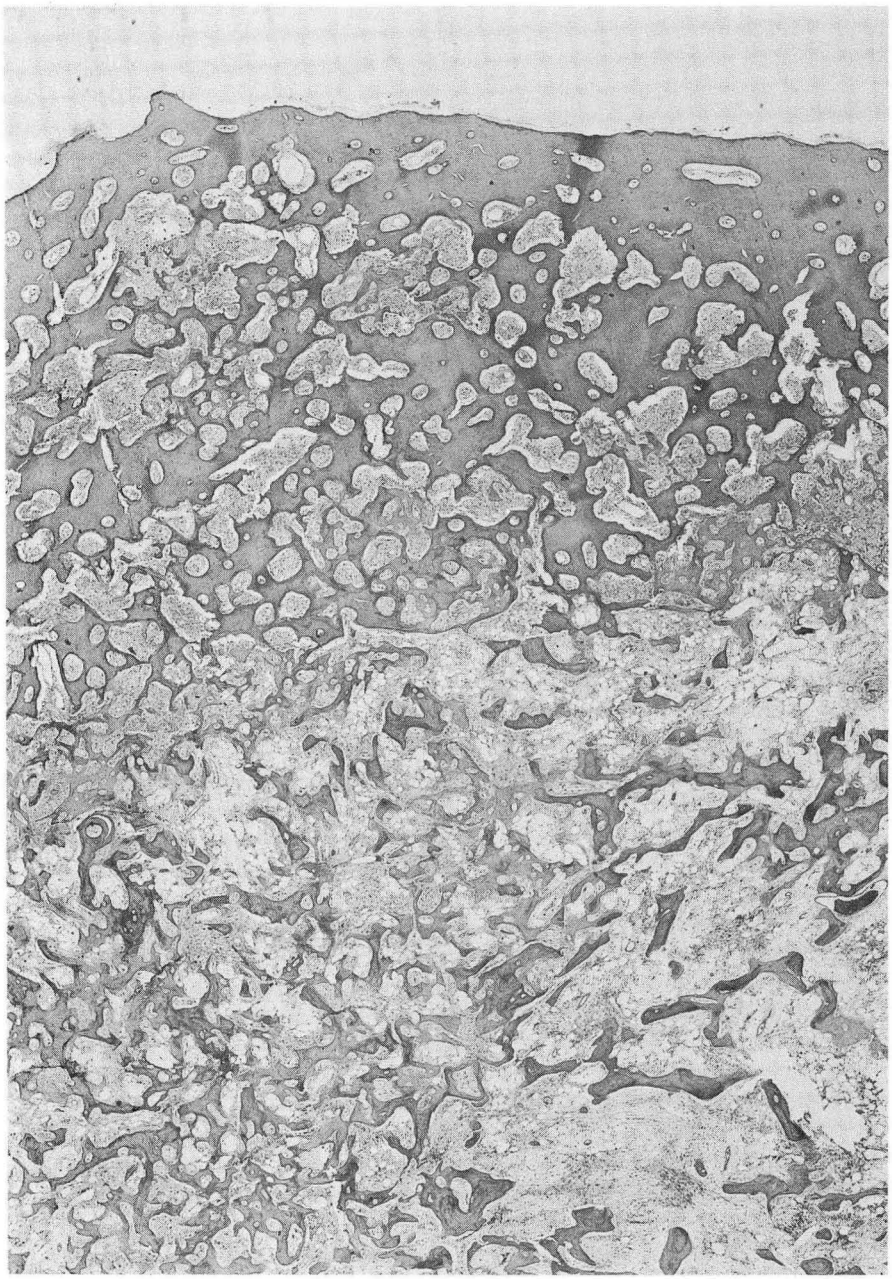
Appearance of label in operated and control femur 1 minute after intravenous injection (7 days postoperative)

Segments	*Counts per segment*	
	Operated	*Control*
	1748	2164
	693	4055
	3	3024
	0	2792
	61	2401
	1432	3773
	5996	5969



DBL 687-5349

Fig. 12



XBB 701-200

Fig. 13

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