

# Histochemical and Autoradiographic Studies on the Effects of Aging on the Mucopolysaccharides of the Periosteum\*

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PLATES 108 TO 110

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## ABSTRACT

An autoradiographic study was made using  $S^{35}$ -sulfate for the localization, distribution, and variation in the mucopolysaccharide content of the femoral periosteum of rats from birth to old age. The mucopolysaccharides were also studied histochemically, using toluidine blue O, Rinehart and Abu'l-Haj's colloidal iron method, and the periodic acid-Schiff reaction, before and after hyaluronidase treatment.

Autoradiograms revealed the uptake of  $S^{35}$  particularly in the vicinity of the preosseous zone and adjacent osteoblasts. This labelling was highest at the period of rapid bone growth. With increasing age, the  $S^{35}$  uptake became progressively less. The preosseous zone showed  $\gamma$ -metachromatic staining at all ages after treatment with toluidine blue. Active osteoblasts were mostly orthochromatic, however,  $\beta$ -metachromasia was exhibited at a later age. Abundant amounts of intra- and extracellular mucopolysaccharides of both the acid and neutral type were demonstrated in the periosteum.

$S^{35}$  uptake and  $\gamma$ -metachromasia show the presence of sulfated mucopolysaccharides, of which chondroitin sulfate predominates in the preosseous zone. Since  $S^{35}$  uptake is high in active osteoblasts, the inability to demonstrate metachromasia in osteoblasts may indicate either that chondroitin sulfate is liberated as fast as it is being produced, or that it may be present within the cells in a precursor form not detectable by histochemical methods.

Numerous investigators using  $S^{35}$  have shown that the isotope becomes incorporated in chondroitin sulfate of bones and cartilage (Dziewiatkowski *et al.*, 1949; Boström and Mansson, 1952; Dziewiatkowski *et al.*, 1957 and Johnston and Comar, 1957). It was pointed out by Dziewiatkowski (1951), that in the diaphysis of long bones of rats, localization of  $S^{35}$  occurred at the periosteal region. Vincent (1954) also indicated that  $S^{35}$  is

fixed in regions of recently formed osseous tissue which has not yet been mineralized. Autoradiographic studies in the anlage of long bones of the chick embryo, revealed radiosulfate deposition apparently at the level of the periosteum and perichondral bone trabeculae (Amprino, 1956).

Although the major composition of bone sulfated mucopolysaccharide is chondroitin sulfate, Meyer (1956, 1958) has found another sulfated polysaccharide, keratosulfate, and a non-sulfated polysaccharide, hyaluronic acid. Sulfated acid mucopolysaccharides are believed to serve as the linkage between the fibrous and crystalline elements or the "local factors" in initiating calcification (Leblond *et al.*, 1955; Sobel, 1955). On the other hand, it has also been suggested that sulfated mucopolysaccharide may serve to maintain an

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uncalcified condition in various areas (Logan, 1935; Haas, 1943, and Sylven, 1947 *a, b*).

Very little work has been reported concerning the biochemical contribution and relationship of the periosteum to the physiology of bone growth, development, and particularly aging. Previous reports by Tonna (1958 *a, b*; 1959) have indicated that this structure is particularly adapted for the study of bone physiology, and although acid and alkaline phosphatases are not altered appreciably by aging, respiratory enzyme activities change considerably. The apparent important role played by mucopolysaccharides in the biochemistry of bone and cartilage pose, from the point of view of the periosteum, a number of pertinent questions, namely: 1. What is the distribution and localization of the sulfated and non-sulfated acid mucopolysaccharides and neutral mucopolysaccharides? 2. What changes occur in the distribution and concentration of these substances during aging? 3. What roles do the osteoblasts play with reference to production, storage, and liberation of mucopolysaccharides during the life of the animal?

The object of this investigation was to obtain further information about mucopolysaccharides during the processes of skeletal aging.

#### *Materials and Methods*

Sprague-Dawley strain rats were segregated into six age groups. Each group was made up of 3 males and 3 females. Group I consisted of 1 week-old rats, Group II of 5 weeks-old, Group III of 8 weeks-old, Group IV of 26 weeks-old, Group V of 52 weeks-old, and Group VI of 104 weeks old rats.

Sulfuric acid-S<sup>35</sup>, diluted with sterile physiological saline solution, was given in two subcutaneous injections to each animal (4  $\mu$ c./gram of body weight per injection), 12 hours apart.<sup>1</sup> Five hours after the last injection, the animals were sacrificed with ether. Immediately after death, cross-sections of bone were taken from the mid-femoral region of both legs and placed in a 3:1 mixture of absolute ethyl alcohol and glacial acetic acid for 2 hours followed by an additional 24 hours of fixation in formol saline and washing for 24 hours (Pelc and Glücksmann, 1955). Paraffin sections were prepared without prior decalcification.

Autoradiograms were prepared using British Kodak AR-10 stripping film and placed in a cold, dry atmosphere as suggested by Messier and Leblond (1957). Emulsions were exposed for 40 days after which time the sections were developed, dehydrated, cleared, and

covered. One set of sections were treated with Feulgen stain prior to covering them with stripping film (Glick, 1949); another set was stained with metanil yellow-iron hematoxylin after developing (Simmel, Fitzgerald, and Godwin, 1951).

Data on the length of the femora (as measured with a pair of calipers from the greater trochanter to a horizontal line with the lateral and medial condyles) were compared with the uptakes of sulfate as assessed by an examination of the autoradiograms.

The following histochemical methods were employed:

1. Toluidine blue O method for metachromasia (Ham and Harris, 1950).
2. Rinehart and Abu'l-Haj colloidal iron method (1951).
3. Periodic acid-Schiff method of Hotchkiss (1948), and
4. Hyaluronidase treatment prior to the application of the histochemical procedures.

In the toluidine blue method, loss of metachromasia was minimized or eliminated by the use of a mixture of equal parts of 5 per cent ammonium molybdate and 1 per cent ferrocyanide and acetone instead of alcohol, for the dehydration of the sections. They were immersed in this dehydrating mixture for 2 minutes.

The Schiff reagent was prepared by the method of Barger and DeLamater (1948). In order to increase the specificity of the reaction, additional slides from all groups were treated with a mixture of 10 per cent acetic anhydride in dry pyridine for 1 hour at room temperature to induce acetylation of the 1:2 glycol groups responsible for the PAS reaction (Pearse, 1950). Another set of slides, after treatment with acetic anhydride-pyridine mixture, were treated with 0.1 N KOH for 1 hour, to reverse the process of acetylation and thus color with PAS reagent so that a better comparison can be made between the 1:2 glycol group distribution and non-specific staining, (McManus and Cason, 1950). Diastase<sup>2</sup> control sections incubated at 37°C. for 2 to 3 hours were also used to eliminate glycogen staining.

Representative sections of all groups were treated prior to toluidine blue O, colloidal iron, and periodic-Schiff staining with a 0.1 per cent testicular hyaluronidase (wydase, 150 TRU/mg.)<sup>3</sup> in physiological saline for 4 hours at 37°C. (Greulich and Friberg, 1957).

The quantitation of results was based on a visual inspection and evaluation of the number of grains per unit area in the autoradiograms and the intensity of the precipitate or staining reactions in the histochemical sections. Comparisons were always made with the results found in the sections of the animals of Group I.

<sup>2</sup> Obtained from Coleman and Bell Co., Norwood, Ohio.

<sup>3</sup> Wydase is the trade name for a lyophilized testicular hyaluronidase prepared by Wyeth Inc., Philadelphia.

<sup>1</sup> Obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee, as carrier-free H<sub>2</sub>S<sup>35</sup>O<sub>4</sub> in 0.2 N HCl.

TABLE I  
*Comparison of  $^{35}\text{S}$ -Sulfate Uptake, Colloidal Iron, and Periodic Acid-Schiff Reactions of the Femoral Periosteum of Rats of Various Ages\**

Animal group.....	I	II	III	IV	V	VI
Age, wks.....	1	5	8	26	52	104
$^{35}\text{SO}_4$ Region of preosseous zone and adjacent osteoblasts.....	++++	+++	++	>+	+	±
R-A Osteoblastic cells.....	++	++++	+	+	±	±
Preosseous zone.....	-	-	-	-	-	-
Periosteal matrix.....	-	-	-	-	-	-
Fibroblastic cells.....	+	++	+	±	±	±
PAS Osteoblastic cells.....	+	++	±	±	±	±
Preosseous zone.....	±	±	±	±	±	±
Periosteal matrix.....	+	++	++	++	++	++
Fibroblastic cells.....	±	±	±	±	±	±

\* Intensity of reaction is indicated by - to +++++.

## RESULTS

### *Autoradiographic Observations:*

Radiosulfate activity appeared to be localized predominantly at the periosteum at all ages. It was concentrated in the area about the preosseous zone and the adjoining rows of osteoblasts.

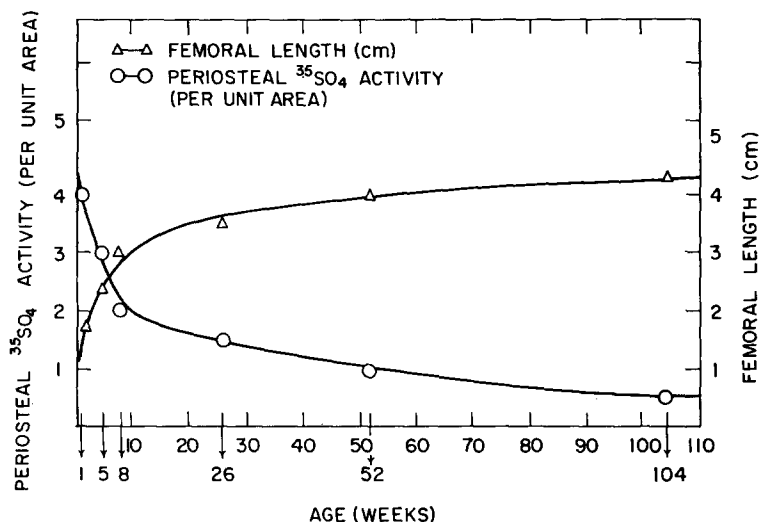
The lateral cortical surface of the cross-section of the femur revealed greater osteogenic activity than the opposite surface and greater uptake of radiosulfate. The periosteum around the gluteal tuberosity also revealed intense  $\text{S}^{35}\text{O}_4$  uptake which was seen in the old as well as in the young animals. Radioactivity was also seen throughout the fibrous layer of the periosteum and regions of calcified bone. At the periphery of newly formed trabeculae, the associated osteoblasts and Haversian canals, the  $\text{S}^{35}\text{O}_4$  content was higher. Many areas contained trabeculae and Haversian systems which did not reveal any increased uptake; however, some "hot spots" were seen. Numerous grains were found in the autoradiograms which corresponded to the osteocytes, the periphery of their lacunae, or both.

As one scanned the autoradiograms from newborn to the 104 week-old rats, the progressive reduction in  $\text{S}^{35}\text{O}_4$  uptake was clearly evident (Table I). This decrease is the mirror image of femoral growth (Text-figs. 1 and 2). The greatest activity was seen in the newborn animals (Fig. 1). At 5 weeks of age the  $\text{S}^{35}\text{O}_4$  uptake was less than that of the younger group. This was also true of

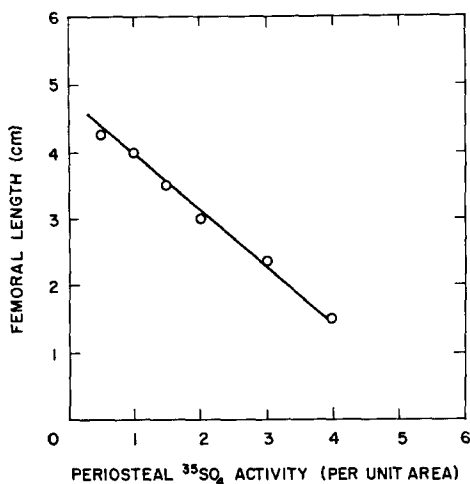
8 weeks-old rats after which time the reduction in radiosulfate uptake was not so dramatic (Fig. 2). In the oldest animals examined, the preosseous zone was virtually missing over long distances of the bone surfaces. Only in occasional areas and for very short distances, was a thin zone noticeable. These animals, nevertheless, exhibited their greatest  $\text{S}^{35}\text{O}_4$  activity in the areas of the preosseous zone and adjacent osteoblasts which have long since become fibroblast in appearance. The level of activity at the peritrabecular surfaces was much less in the older animals.

### *Histochemical Observations:*

1. *Toluidine Blue Studies.*—Orthochromatic staining of the osteoblasts, and occasionally some  $\beta$ -metachromasia was seen. In a few well defined active osteoblasts of newborn rats, the cytoplasm making contact with the preosseous zone revealed what appeared to be  $\gamma$ -metachromasia. The adjoining preosseous zone gave an intense reddish-pink ( $\gamma$ -metachromatic) color (Fig. 3). The fibroblasts and fibrous elements of the periosteum revealed  $\beta$ -metachromasia. Eight week-old animals exhibited a similar picture. As the animals became older there was a shift in the staining of osteoblasts from a predominant orthochromatic to  $\beta$ -metachromatic staining. This change paralleled their change in shape to fibroblastic spindle-shaped cells. The preosseous zone, as long as it was evident, was  $\gamma$ -metachromatic. The endosteum revealed a  $\gamma$ -metachromatic line similar in appearance to the



TEXT-FIG. 1. Comparison between the growth in the femoral length (*cm.*) and  $^{35}\text{S}$ -sulfate activity of the femora periosteum of rats at various ages. The activity values are given in arbitrary units and represent a given number of reduced grains in the autoradiograph per unit area of periosteum.



TEXT-FIG. 2. A plot representing the increment of femoral growth in length against the increment of  $^{35}\text{S}$ -sulfate activity given in Fig. 1. The straight line reveals a good mirror image approximation between these parameters.

preosseous zone of the external cortical surface (Fig. 4 and Table II).

Treatment with testicular hyaluronidase removed the  $\gamma$ -metachromasia of the preosseous zone.

2. *Colloidal Iron Studies.*—This histochemical method gave a blue reaction with mucopolysaccharides and revealed an abundance of these substances associated with osteoblasts, especially

TABLE II

*Metachromatic Variations Exhibited by the Femoral Periosteum of Rats at Different Ages by Toluidine Blue O\**

Periosteal components	Age, wks.					
	1	5	8	26	52	104
Periosteal matrix. . . . .	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
Fibroblastic cells. . . . .	$\beta$	$\beta$	$\beta$	$\beta$	$\beta$	$\beta$
Osteogenic cells. . . . .	$\alpha$	$\alpha, \beta$	$\beta$	$\beta$	$\beta$	$\beta$
Preosseous zone. . . . .	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$

\* Orthochromasia =  $\alpha$  (monomer); Metachromasia =  $\beta$  (dimer) or  $\gamma$  (polymer)

those nearest to the preosseous zone in Group I animals. The preosseous zone was negative. Other cells of the periosteum, including fibroblasts and osteogenic cells, were also positive. The blue staining material occurred as a precipitate about the surface of the cells (Fig. 5). Strands of this blue amorphous reacting product were seen to bridge cells and also appeared in irregular masses about the fibrous elements of the periosteum. This reaction is not entirely an extracellular one because definite blue staining short rods and granules could be seen within the cytoplasm of the cells (Fig. 5). These structures, reminiscent of the mitochondria, were most abundant in osteoblasts. The amorphous precipitate type of reaction appeared to occur only extracellularly.

At 5 weeks of age there was an increase of intracellular rods and granules. The sections from 8 week-old rats revealed a mucopolysaccharide content similar to that of the newborn; however, a steady low-content persisted in the later ages. The preosseous zone was negative except for an occasional blue color about the edges of the newly calcified bone. Large pools of deep blue products were seen collected at various areas throughout the cortical bone. This occurred especially around newly forming trabeculae and some lacunae. These areas were most abundant in younger animals but also were seen in the oldest animals examined. The colloidal iron reaction of the periosteum at different ages is shown in Table I.

Hyaluronidase treatment removed the extracellular material which gave the blue reaction; the intracellular rods and granules, however, persisted.

3. *Periodic Acid-Schiff Studies.*—The cells of the periosteum of newborn rats, especially the osteoblasts, gave a positive reaction after PAS. This reaction, however, was entirely cytoplasmic. All the fibrous elements of the periosteum were similarly positive. At 5 weeks of age the osteoblasts gave a deeper color, which was also given by the fibrous matrix. The following ages gave a faint cellular reaction, but the fibrous elements appeared more intense. The preosseous zone revealed only a very faint color throughout the six age groups. Amorphous, red cytoplasmic granules seen in some osteoblasts were removed by diastase treatment. The PAS reaction of the periosteum at various ages is given in Table I. Hyaluronidase treatment did not remove the positive reaction after PAS staining.

#### DISCUSSION

The finding of large quantities of bound radiosulfate in the periosteum substantiated the findings of previous investigators (Dziewiatkowski, 1951; and Amprino, 1956). The maximum uptake of radiosulfate appeared even before the maximum rate of calcification. This indicates, perhaps, that the production of sulfated mucopolysaccharides precedes calcification as was indicated by the work of Sobel (1955), Leblond *et al.* (1955), and Amprino (1956).

Throughout the life of the animal the amount of  $S^{35}O_4$  accumulated by the periosteum decreases progressively. This reduction in  $S^{35}O_4$  as chondroitin sulfate, with increasing age has been shown to occur in human costal cartilage by Stidworthy,

Masters, and Shetlar (1958). When the curve representing retained  $S^{35}O_4$  at different ages is compared to the curve of the growth of the femur in length, it is readily seen that these curves appear almost as mirror images. The scatter diagram of the increment of growth versus the uptake of radiosulfate shows a good correlation between the growth of a bone and the quantity of  $S^{35}O_4$  present at the various ages. The reduced uptake of  $S^{35}O_4$  by the periosteum with increasing age, may indicate that less radiosulfate is used at this site since the rate with which bone is being laid down is decreased as the animal ages.

$S^{35}O_4$  autoradiograms indicate a preferential uptake of the isotope by the periosteum even though the large active osteoblasts and the other cells of this tissue show only  $\alpha$  and  $\beta$ -metachromasia after staining with toluidine blue. Since in many cases the osteoblasts lining the preosseous zone show no boundary between the part of the cytoplasm adjacent to the preosseous zone and the preosseous zone itself, it becomes difficult to establish whether these osteoblasts exhibit  $\gamma$ -metachromasia. It is possible that the amount of acid mucopolysaccharide is at a level which can be easily masked by the presence of the orthochromatic material or that it cannot be detected by the histochemical method. Sobel (1955) has indicated that the presence of calcium ions up to a certain concentration brings about a rearrangement of chondroitin sulfate, thereby making it react metachromatically. Beyond a certain concentration of calcium there occurs a gradual decrease in metachromasia.

At our present state of knowledge concerning the production of mucopolysaccharides, it seems likely that these substances or their precursors are manufactured by the osteoblasts and other cells of the periosteum. Unlike chondrocytes, periosteal cells do not appear to store abundant quantities of sulfated mucopolysaccharides since this would have been shown histochemically. The failure of osteoblasts to reveal metachromasia after toluidine blue staining indicates that acid mucopolysaccharides or their precursors are liberated as soon as they are synthesized and that they are synthesized as needed.

The position of the osteoblasts with relation to the preosseous zone is interesting from a biochemical point of view. If we assume that the large sulfated mucopolysaccharides and collagen form an integral part of the structure of the organic matrix of bone prior to its calcification,

then the position and distance of the osteoblasts from the preosseous zone are important. Wherever osteoblasts are found, they generally tend to accumulate in rows about bone surfaces. Their strategic location can be envisioned as necessary for the laying down of the fibrous matrix in the proper position, as a telephone cable is unreel from its spool. Fitton Jackson and Randall (1956) suggested that, in the formation of protein and its associated non-sulfated mucopolysaccharide, the latter is sulfated as it is liberated by the cell.

Colloidal iron staining of the periosteum indicated the presence of an abundant quantity of extracellular acid mucopolysaccharide about the periosteal cells, especially the osteoblasts, which was removed by hyaluronidase treatment. The preosseous zone exhibited  $\gamma$ -metachromasia after toluidine blue staining and failed to give a positive reaction with colloidal iron. Since acid mucopolysaccharide is known to be present in the preosseous zone, the molecules in this region appear to exist in a masked state, so that they are unable to react with colloidal iron. Perhaps the association of collagen with chondroitin sulfate or the presence of calcium salts at nucleation centers mask this reaction. Levine *et al.* (1949) pointed out that fully mineralized bone stained faintly or not at all.

The reduction in staining intensity seen in older animals was similar to that found for human costal cartilage by Joel, Masters, and Shetlar (1956) using Hale's colloidal iron method (Hale, 1946).

Hyaluronidase treatment, which removed the extracellular colloidal iron staining reaction, had no effect upon the intracellular findings. The intracellular particulates which stained blue after colloidal iron treatment were found in all the cells of the periosteum, especially in active osteoblasts. In osteoclasts the number of these particles was so high that they could not be counted. Changes in the number of these intracellular structures with age and cellular activity parallel what was seen in recent mitochondrial studies (Tonna and Pillsbury, 1959 *a, b*). It is believed therefore, that the mitochondrial complement of these cells is being stained. However, the staining may be due to non-specific "adsorptive" binding.

Granules exhibiting a positive reaction after PAS seen in periosteal cells appeared to be similar to the glycoprotein granules described by Heller-Steinberg (1951). At the period of intense calcification and bone growth, this muco- or glyco-

protein was most abundant. Whether the granules are in any way metabolically associated with sulfated mucopolysaccharides is not known; however, it is felt that they are involved with the makeup of the ground substance.

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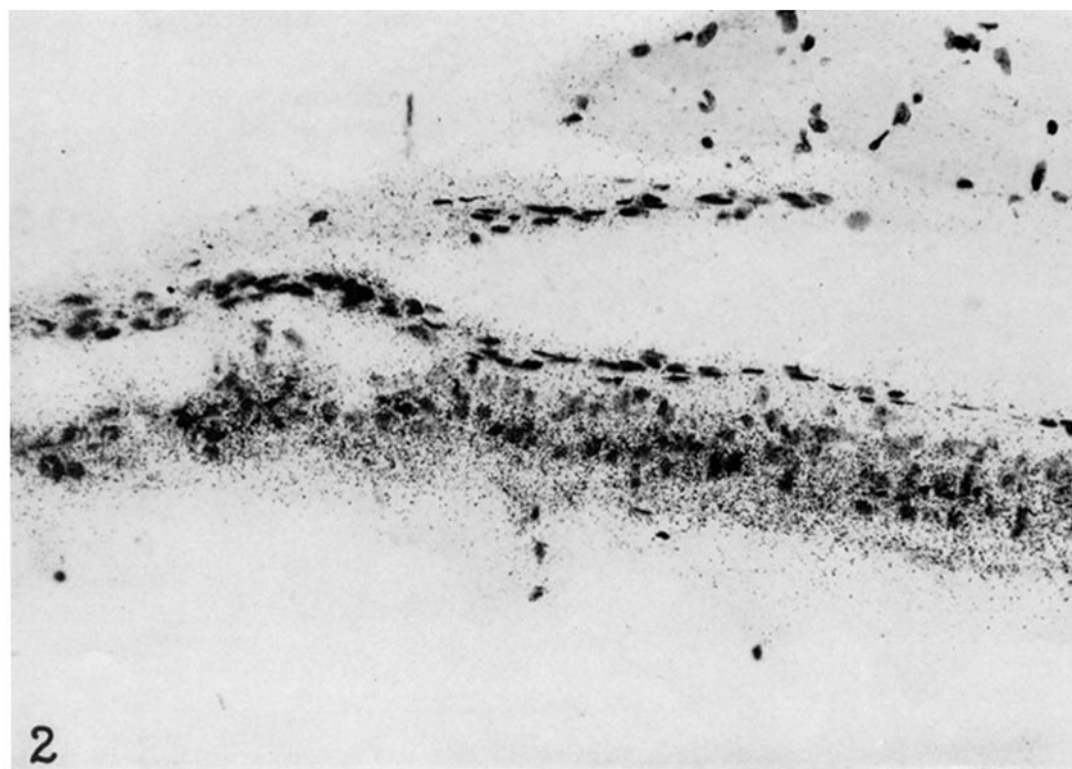
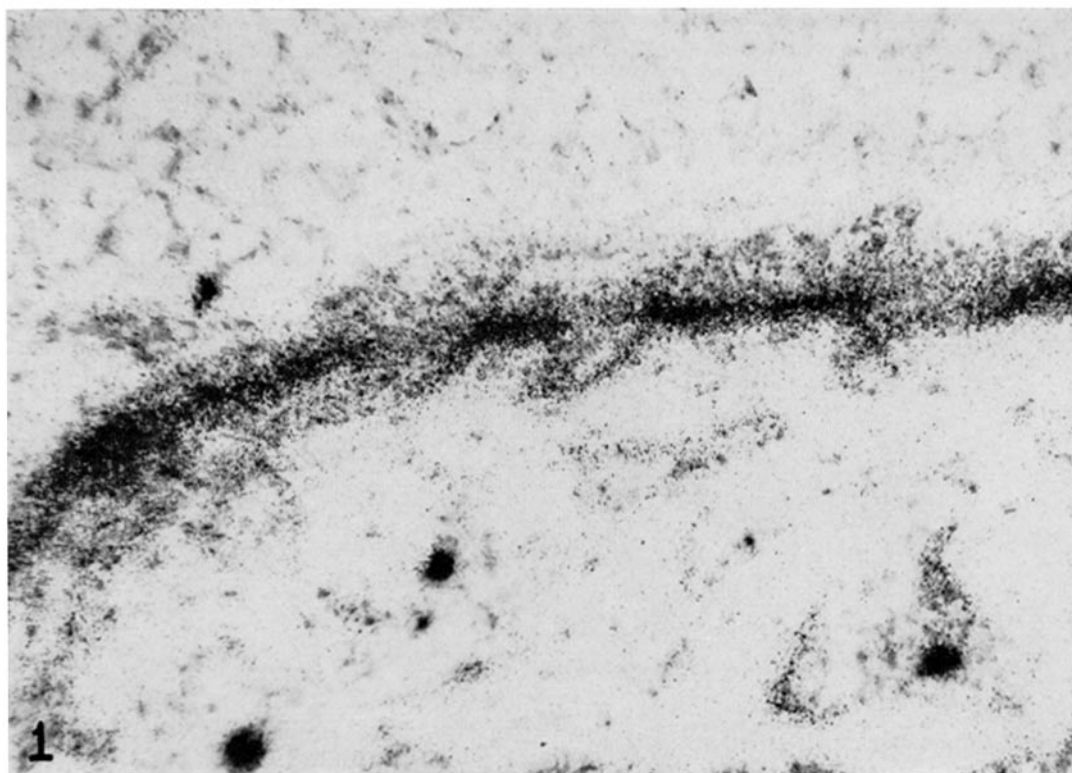
## EXPLANATION OF PLATES

## PLATE 108

FIG. 1. A  $^{35}\text{S}$ -sulfate autoradiograph of developing femoral cortical bone and adjacent periosteum of a newborn rat. The greatest uptake is seen along the periosteum, and especially in the vicinity of active osteoblasts and the preosseous zone. Localized "hot spots" can be seen in the cortical bone.  $\times 563$ .

FIG. 2. A  $^{35}\text{S}$ -sulfate autoradiograph of femoral periosteum and remnants of cortical bone of an 8 weeks-old rat. The uptake of radiosulfate appears much reduced at this age as compared to that exhibited by the periosteum of newborn animals.  $\times 563$ .



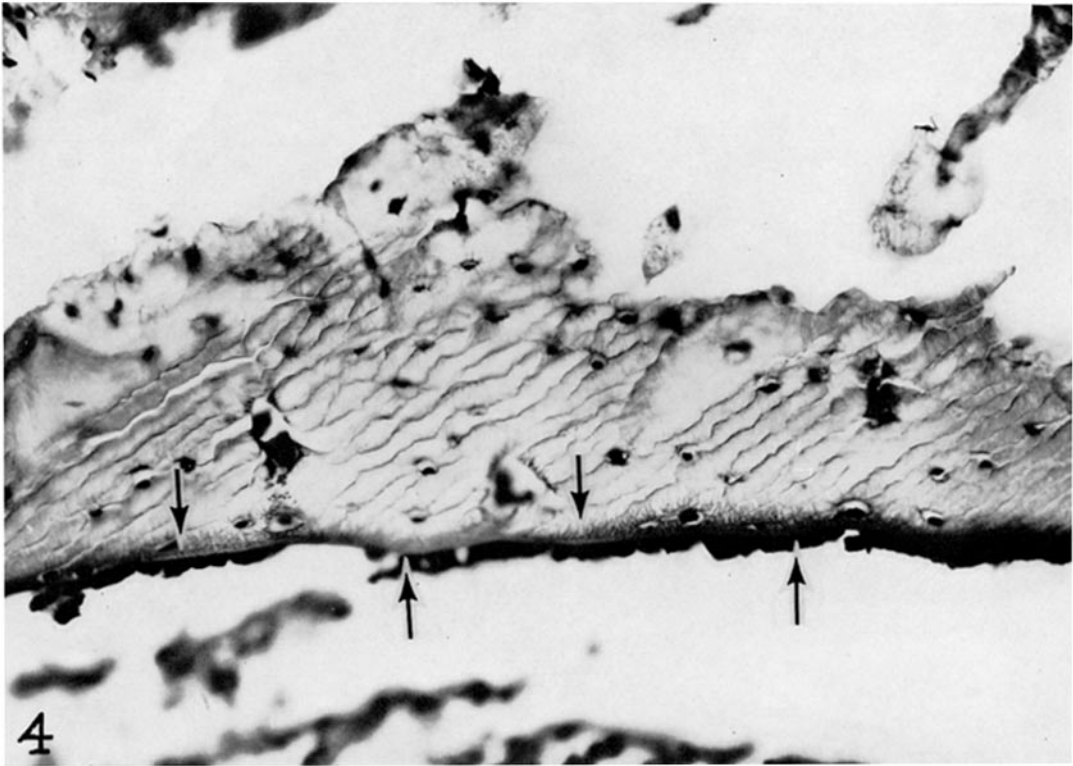
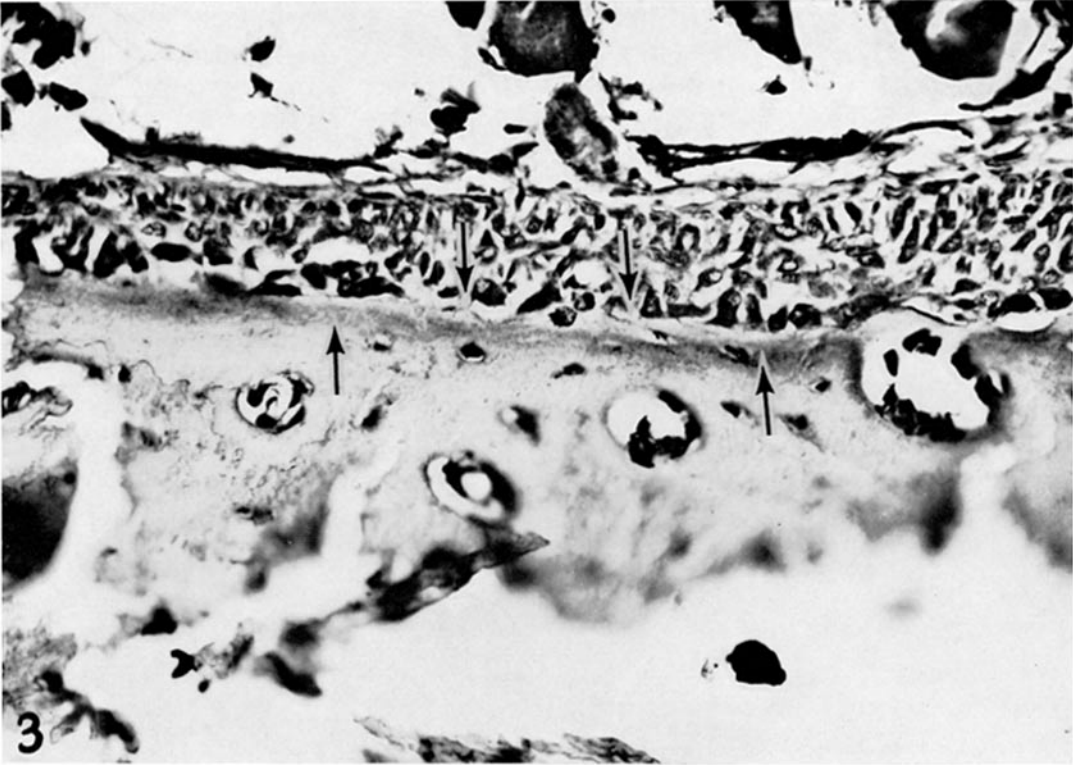


(Tonna and Cronkite: Studies on effects of aging)

PLATE 109

FIG. 3. A cross-section of femoral cortical bone and adjacent intact periosteum of an 8 weeks-old rat stained for metachromasia with toluidine blue. The clear  $\gamma$ -metachromatic proosseous zone (arrows) is noticeable between the deeply stained osteoblasts and cortical bone.  $\times$  563.

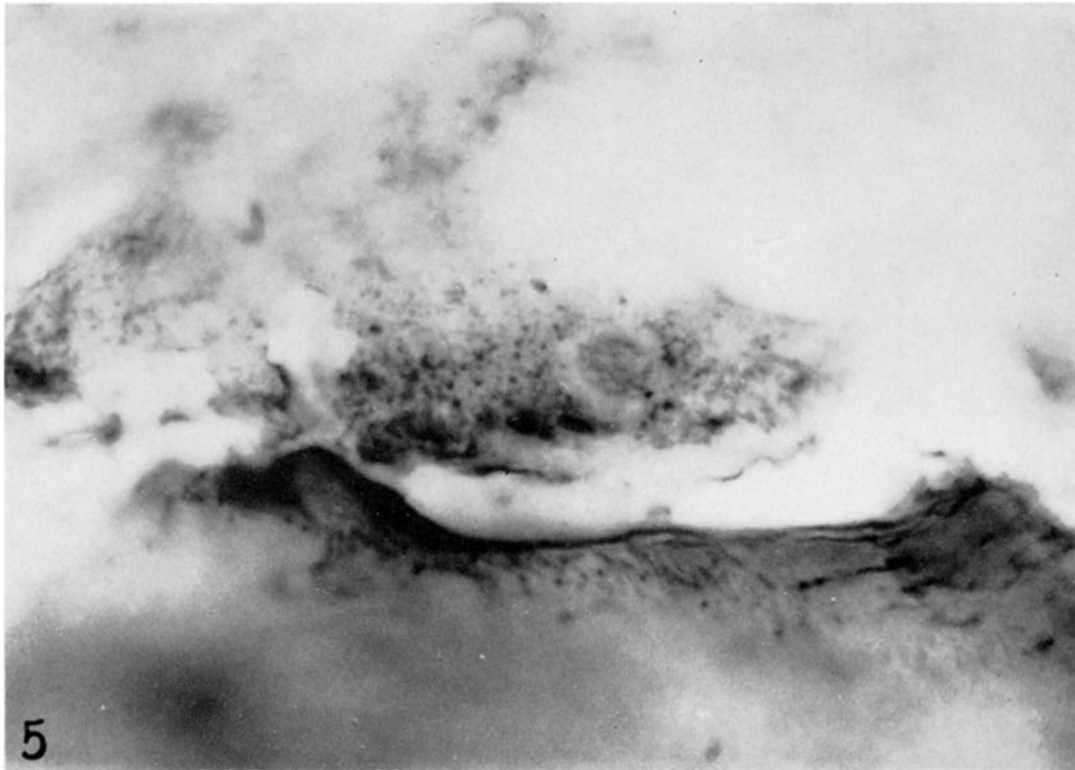
FIG. 4. A cross-section of the endosteal side of femoral cortical bone of a 5 weeks-old rat stained for metachromasia with toluidine blue. A thin clear  $\gamma$ -metachromatic zone (arrows) is seen between the deeply stained endosteal cells and cortical bone.  $\times$  563.



(Tonna and Cronkite: Studies on effects of aging)

PLATE 110

FIG. 5. The photomicrograph reveals a periosteal osteoblast of the femur of a newborn rat stained for acid mucopolysaccharide by Rinehart and Abu'l-Haj colloidal iron method. The osteoblast exhibits numerous intracytoplasmic colloidal iron-positive bodies.  $\times 880$ .



(Tonna and Cronkite: Studies on effects of aging)