THE CELLULAR COMPLEMENT OF THE SKELETAL SYSTEM STUDIED AUTORADIOGRAPHICALLY WITH TRITIATED THYMIDINE (H^3TDR) DURING GROWTH AND AGING

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ABSTRACT

An autoradiographic study has been made of mouse femora from birth to one year of age. Tritiated thymidine was used to study the proliferative rate of various skeletal tissues. The labeling index of the periosteum was found to be highest at birth (0.085). At 8 weeks of age the index fell sharply to 0.007 and remained low throughout the entire period of study. Chondro-osteogenic cells of the periosteum, especially those at the perichondrial zone, were most frequently labeled initially. The labeling index of the epiphyseal disk remained high (0.054 to 0.048) until about the 6th week of age. Following this age the index fell sharply to 0.018 at 8 weeks and 0.002 at 26 weeks of age. Autoradiographs showed that the cells of the articulating surface of the epiphysis and disk are derived at least in part from migrating chondro-osteogenic cells of the periosteum residing at the perichondrial region, and that these cells serve as the progenitor pool necessary for both circumferential growth and expansion of the epiphyseal disk.

An understanding of skeletal aging requires knowledge of the functional as well as of the morphological changes with time in the cells of the skeleton. This point is especially pertinent since many of the morphological changes recognized in old age are represented by some basic alteration in the chemistry of the cell at an earlier period in the life of the organism. One such example is the depression of respiratory enzyme activity which was shown to occur in 8-week-old rats prior to observed cytological age changes (Tonna, 1958*a*, 1958*b*, 1959).

A descriptive morphological analysis of recognized cellular changes coincidental with aging is an important first-line approach to the aging problem. However, this approach is static and must eventually lead to the design of experiments which will furnish dynamic data, if we are to understand an intricate biological system such as is represented by the skeletal system during growth, development, repair, and aging.

In order to obtain a better understanding of bone growth and the phenomenon of skeletal aging, it is important to have knowledge concerning (*a*) the source of cells making up the diaphysis and epiphysis of a long bone, (*b*) the proliferative rate and potential of the progenitor pool, and (*c*) any alterations in the proliferative rate and potential of skeletal cells with aging. It was the object of this investigation to furnish information concerning the changes in the proliferative rate and potential of the cellular complement of the skeletal system during bone growth and aging.

MATERIALS AND METHODS

Experiment I: A total of 45 female mice of a Brookhaven National Laboratory Swiss Albino strain was

used in this experiment. The animals were divided into five age groups, namely, 1, 5, 8, 26, and 52 weeks of age. One hour prior to killing, each mouse received a subcutaneous injection of 0.5 μc. of tritiated thymidine[1] per gram of body weight.

Experiment II: 20 female mice, 5 weeks of age, were given a similar dose of isotope and were killed from 1 hour to 25 days following isotope administration.

In both experiments the femora were removed intact from each animal and fixed with acetic-alcohol for 3 hours, followed by an additional 24 hours of fixation in formol-saline and washing for 24 hours (Pelc and Glücksmann, 1955). Decalcification was carried out in a 10 per cent solution of Versene. Deparaffinized tissue sections were covered with British Kodak AR-10 stripping film and exposed for 20 days in a cold dry atmosphere. The preparations were stained with Harris hematoxylin after developing. Additional tissue sections were prepared and stained routinely with hematoxylin and eosin.

Interpretation of tritiated thymidine autoradiographic data was based on previous reports by Hughes *et al.* (1958), Cronkite *et al.* (1959*a*, 1959*b*), Bond *et al.* (1959), and Quastler and Sherman (1959).

The periosteal and epiphyseal disk populations were studied with a Whipple disk. At the periosteum only cells of the osteogenic layer were counted. Femora were scanned from the mid-diaphyseal region to and including the perichondrial zone. Cells which possessed cartilage cell characteristics were not included in the periosteal counts. A periosteal labeling index was obtained by dividing the number of labeled cells by the total population. This index represents a count of pre-osteoblastic and osteoblastic cells plus a large pool of labeled osteogenic cells of the perichondrial region. Cells making up the proliferating and hypertophic zones of the epiphyseal disk were counted and the labeling index was obtained.

RESULTS

Experiment I: Osteogenic cells of the periosteum were found predominantly labeled 1 hour after administration of tritiated thymidine (Fig. 3). Occasionally osteoblasts were seen labeled. On the other hand, fibroblasts of the fibrous periosteal layer were rarely labeled. The largest population of labeled cells was seen at the perichondrial region of the periosteum, especially the posterior aspect of the femur of young animals (Fig. 4). Frequent labeling of osteogenic cells lining the trabeculae of spongy bone was seen.

At the distal half of the femur in 1-week-old mice, a large proportion of the periosteal population was found labeled (8.5 per cent). The percentage of labeling diminished to approximately one-third at 5 weeks of age (2.7 per cent). By 8 weeks of age the labeling was 0.7 per cent. This value did not vary significantly during the following ages (Table I; Fig. 1).

Autoradiographs revealed that in young animals a large percentage of the population of osteogenic cells of the perichondrial zone, extending into the periphery of the epiphyseal disk, was labeled. Chondroblasts at the zone of resting

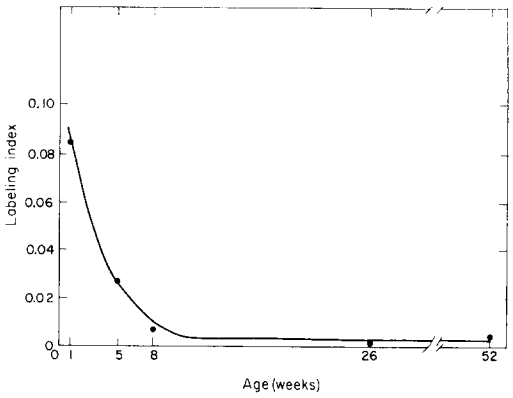


FIGURE 1

A plot representing the labeling index (total number of labelled cells per total population) of the osteogenic layer of mouse femoral periosteum studied at various ages. Note that from birth to 8 weeks of age a considerable change occurs in the proliferative capacity of the periosteum.

[1] Obtained from Schwarz Bioresearch Inc., Mt. Vernon, New York.

TABLE I

Tritiated Thymidine–Labeled Cellular Population of Osteogenic Layer of Distal Half of Mouse Femoral Periosteum

Age (weeks)	1	5	8	26	52
Labeling index	0.085	0.027	0.007	0.002	0.006
% labeling	8.5	2.7	0.7	0.2	0.6
No. cells counted	2666	2312	1181	1115	553

cartilage were not labeled. However, labeling was seen at the proliferative and hypertrophic zones of the epiphysis (Figs. 5 and 6). An estimation of the percentage of labeling of the population of the proliferative and hypertrophic zones at various ages is given in Table II (see Fig. 2). One week after birth, 5.4 per cent of the cells making up the proliferative and hypertrophic zones were labeled. This labeling was approximately the same at 5 weeks of age (4.8 per cent). Between 5 and 8 weeks of age the labeled population decreased to one-third of the original value (1.8 per cent). By 26 weeks of age only 0.2 per cent of the population was seen labeled. No labeling was encountered at the epiphyseal disk at 52 weeks of age.

Experiment II: A study of autoradiographs taken at intervals between 1 hour and 25 days revealed a migration of labeled cells from the perichondrial region of the periosteum to the articulating surface of the distal femur and into the epiphyseal disk. This migration appeared at both the anterior and posterior surfaces of the femur (Figs. 7 and 8). However, migration was more evident at the posterior surface. Cartilage cells adjacent to the perichondrial zone were labeled. Labeling could be traced along the articulating surface up to about 10 days (Figs. 9 and 10). Following this period sufficient cell divisions had taken place so that the label became less detectable. The labeled population of the perichondrial zone became diminished as the labeled cells migrated away from this site.

Labeled cells which extended into the epiphyseal disk could be traced from the zone of resting cells in this experiment to the zone of degeneration (Fig. 11). This was observed 2 days following the administration of the isotope. At about 10 days labeled cells were rarely encountered. From 12 days on, no labeled cells were seen at the epiphyseal disk.

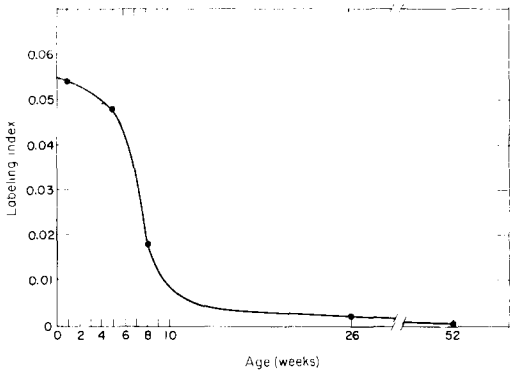


FIGURE 2

A plot representing the labeling index of the distal femoral epiphyseal disk of mice studied at various ages. Note the considerable reduction which occurs in the proliferative capacity of the disk cartilage cells between 5 and 10 weeks of age. The proliferative capacity of periosteal cells is already low at a time when the disk cell capacity is still high. It is believed that this plot represents a unique proliferative capacity of disk cells which have been originally derived from periosteal cells.

DISCUSSION

Thymidine, thymine desoxyriboside, is incorporated into DNA or promptly degraded (Hughes, 1958; Rubini *et al.*, 1960). Following the incorporation of tritiated thymidine into DNA, it is diluted only by successive cell divisions, since there is no significant exchange of the tritium in thymine or of thymine in DNA. Accordingly cells labeled with this material can be studied with respect to decrease in the intensity of the label as mirrored by the average grain count of the cell, the percentage of cells labeled, and their migration pathways. The data thus obtained make it possible to study not only the proliferative capacity but the total life cycle of the given cell. Tritium is very

TABLE II

*Tritiated Thymidine–Labeled Cellular Population of Femoral Distal Epiphyseal Disk of Mice**

Age (weeks)	1	5	8	26	52
Labeling index	0.054	0.048	0.018	0.002	0
% labeling	5.4	4.8	1.8	0.2	0
No. cells counted	1493	1784	1889	1507	1432

* Only cells making up the proliferative and hypertrophic zones were counted.

desirable for autoradiography because of its high resolution. The high resolution results from the low energy of the beta emission, maximum 0.018 Mev, average 0.006 Mev. The maximum path length is of the order of 1 micron, producing the excellent resolution of the silvergrains in the overlying autoradiographic preparation (Robertson and Hughes, 1959). The metabolic and labeling studies of Rubini *et al.* and Cronkite *et al.* indicate that the effective availability time for intravenously administered tritiated thymidine in man is of the order of 20 minutes or less. Labeling of cells was found as early as 1 minute after injection. The percentage of labeling and the mean grain count of a uniform population of cells such as the basophilic normoblast were noted to climb at a decreasing rate for approximately 60 minutes. Effectively the labeling process was complete within about 20 minutes. In all tissues studied so far, the time for DNA synthesis in mammalian tissues is apparently between 6 and 12 hours. Since the availability time of the label is relatively short in respect to the time for DNA synthesis, a "flash" labeling index of a proliferating population approximates the ratio of the time for DNA synthesis to the average time between successive mitoses. Problems concerned with the kinetic analysis of cell proliferation following labeling with tritiated thymidine have been considered by Quastler and Sherman (1959) and Cronkite (1959).

During the elongation of long bones and circumferential growth, a large population of cells is required. These cells must come either from dividing or non-dividing pools, perhaps formed during the embryological development of the skeleton, or from a continuously available pool residing outside the skeletal system proper. During the early period of embryogenesis, bones are recognized as condensations of mesenchyme (Gardner, 1956). The fibrous periosteum which appears at a later period surrounds these regions and isolates the mesenchymal cells from the general mesenchymal components and their derivatives. Osteogenic cells differentiate from the mesenchyme in normal intramembranous bone formation and serve as precursors for osteoblasts. In endochondral bone formation cartilage is first differentiated from the mesechyme. With time, bone replaces the cartilage. Pre-osteoblasts beneath the fibrous periosteum are capable of proliferating and thus are able to supply the osteoblasts needed for bone formation (Pritchard, 1956).

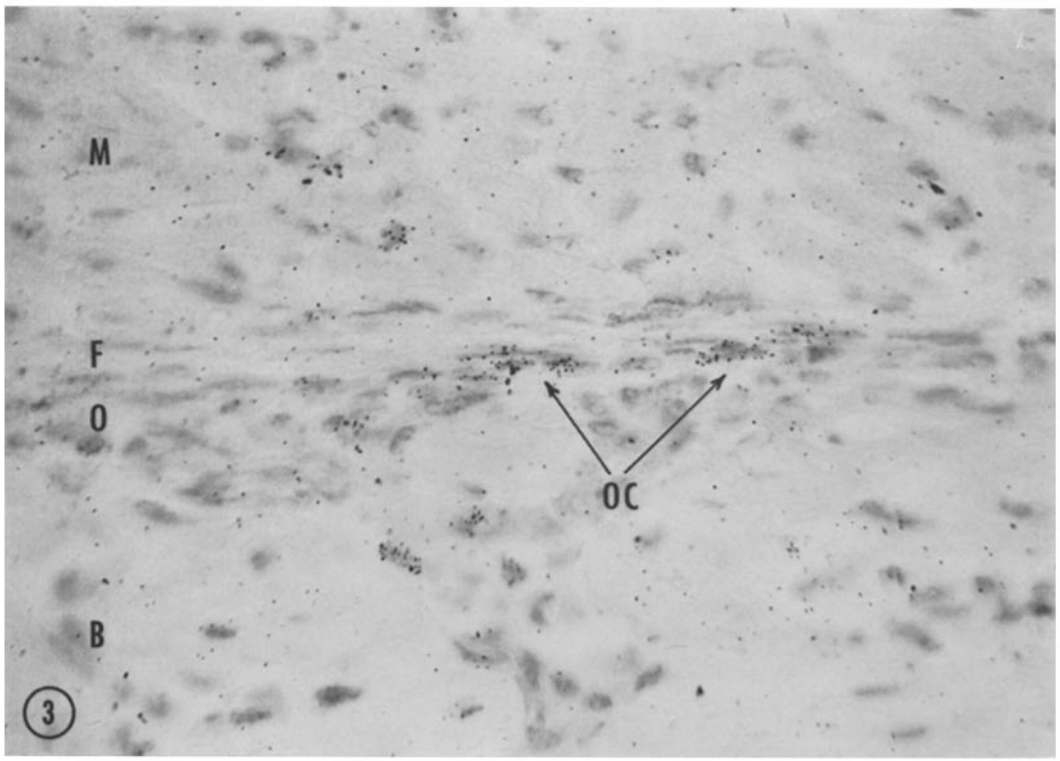
The labeling of mouse femora with tritiated thymidine offers a simple and convenient way of studying the proliferative capacity of various cells and tissues. Results show that the pre-osteoblasts of the periosteum were frequently labeled in young mice, indicating active DNA synthesis. It is interesting to note that occasionally osteoblasts were seen labeled. A high percentage of osteoblastic labeling was observed after fractures in recent studies (Tonna, 1960*a*, 1960*b*). It appears that, at least in young mice, some or all active osteoblasts are capable of dividing. The proliferative rate of osteogenic cells was greatest immediately after birth. Between 1 and 8 weeks of age the proliferative rate diminished rapidly. This change was coincidental with the period of active bone formation. During aging there was a progressive depletion of osteogenic cells which were

FIGURE 3

Autoradiograph of periosteum taken from the mid-diaphyseal aspect of the femur of a 1-week-old mouse. The periosteum is indicated by *F* and *O*. *F* indicates the fibrous layer, and *O* the osteogenic layer. Osteogenic cells can be seen labeled and are designated *OC*. *M* and *B* indicate muscle and cortical bone respectively. Harris hematoxylin stain. Oil immersion. × 414.

FIGURE 4

Autoradiograph of the posterior aspect of the perichondrial region of the distal epiphysis of the femur of a 1-week-old mouse. The chondro-osteogenic cells of the periosteum (*O*) are undergoing active DNA synthesis and are heavily labeled with tritiated thymidine. *C* indicates degenerating cartilage of the epiphyseal disk. The arrow points to the direction of the distal epiphyseal disk. The isotope was given 1 hour prior to sacrificing. Harris hematoxylin stain. Oil immersion. × 414.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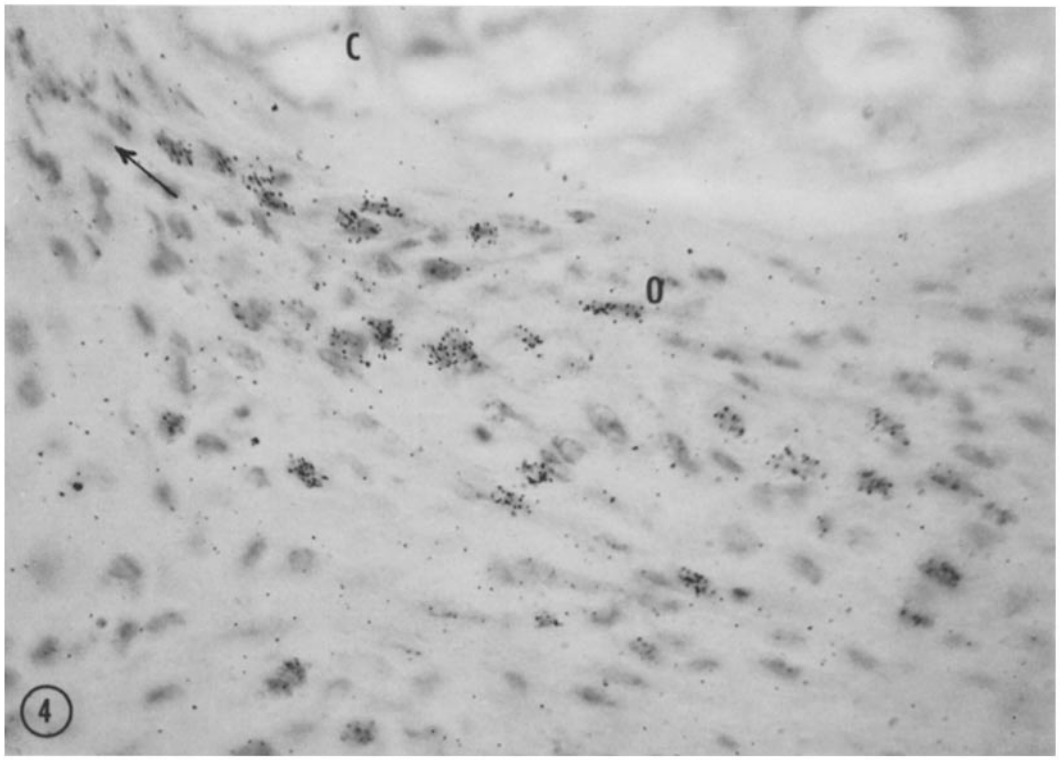
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isolated during early embryonic development. In old animals the osteogenic layer, in almost the entire length of the periosteum, was virtually missing except for a few flattened cells exhibiting pyknotic nuclei.

Leblond and Greulich (1956) are of the opinion that expansion of the epiphysis results from the addition of cartilage cells to the articulating surface by the transformation of fibrocytes into chondrocytes, and that chondrogenic activity at the periphery of the disk produces an increase in the diameter. In the present study labeled cells were rarely seen at the articulating surfaces (see Fig. 9). It was noted in the time studies, however, that the perichondrial cells migrate to the articulating surfaces (Fig. 6). Widening of the epiphyseal disk, on the other hand, resulted from chondrogenic activity at its periphery, especially from the posterior aspect of the femur. These autoradiographic findings indicate that the periosteum is responsible for the supply of cells for diaphyseal and, at least in part, epiphyseal growth. The periosteum is, therefore, indirectly responsible for growth of the epiphyseal disk as well as for appositional growth (Fig. 12). A plot of the labeled disk cells (Fig. 2) indicates that the periosteal supply of cells to the disk is perhaps inadequate to meet the large demand during the period of active longitudinal bone growth. The deficiency appears to be eliminated, however, by the mitotic divisions which take place at the proliferative and hypertrophic zones of the epiphyseal disk. It will be noted that between 1 and 5 weeks of age the proliferative rate of the periosteum was considerably diminished, while the proliferative rate of the epiphyseal disk remained rather high. Beyond 5 weeks of age the proliferative rate of the disk also diminished.

Since the labeling index of the epiphyses diminished with increasing age, it would appear that some cellular alteration had taken place resulting in a reduced turnover rate of cells. These cells spend a considerably longer time at the epiphyseal disk as a result, allowing characteristic age changes to take place. Periosteal osteoblasts are believed to undergo similar changes with increasing age (Tonna, 1958*a*; Tonna and Pillsbury, 1959*a*, 1959*b*; Tonna and Cronkite, 1959).

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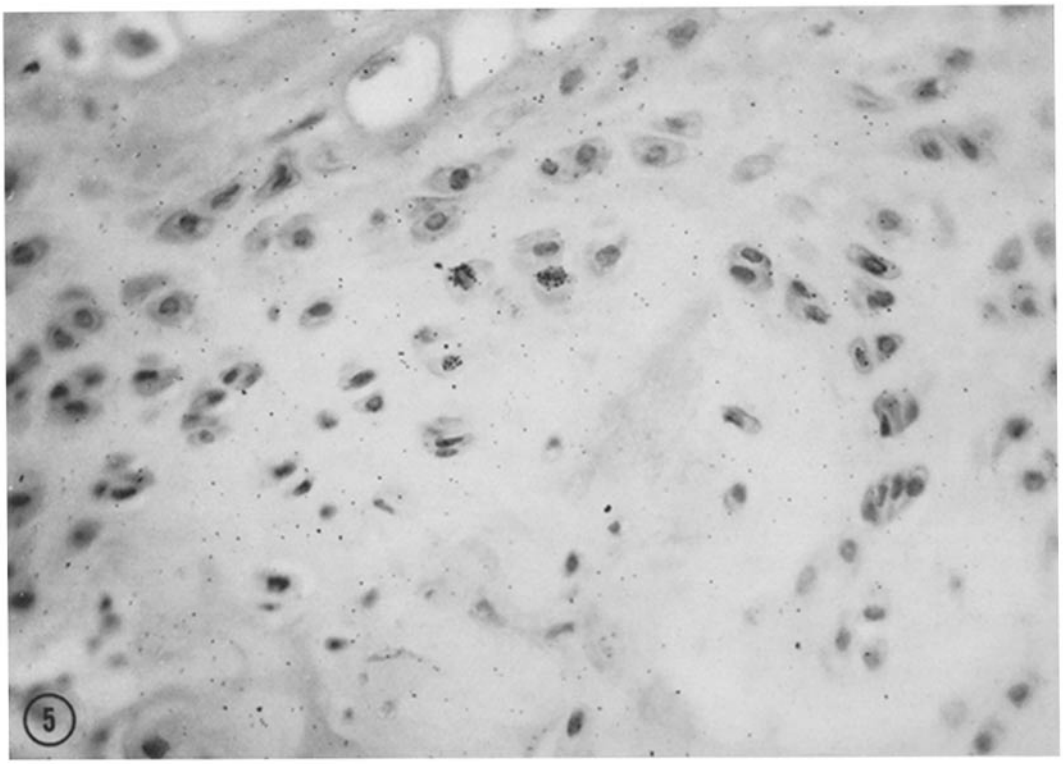
FIGURE 5

Autoradiograph of the distal femoral epiphyseal disk of a 3-week-old mouse. Labeled cells are seen in the proliferative and hypertrophic zones. The animal was sacrificed 1 hour after isotope administration. Harris hematoxylin stain. Oil immersion. × 338.

FIGURE 6

Autoradiograph of the distal femoral epiphyseal disk of a 5-week-old mouse, 1 day after isotope administration. Tritium-labeled thymidine uptake is shown by cartilage cells of the proliferative and hypertrophic zones. The zone of resting cartilage is found above the labeled cells in the photomicrograph. Harris hematoxylin stain. Oil immersion. × 847.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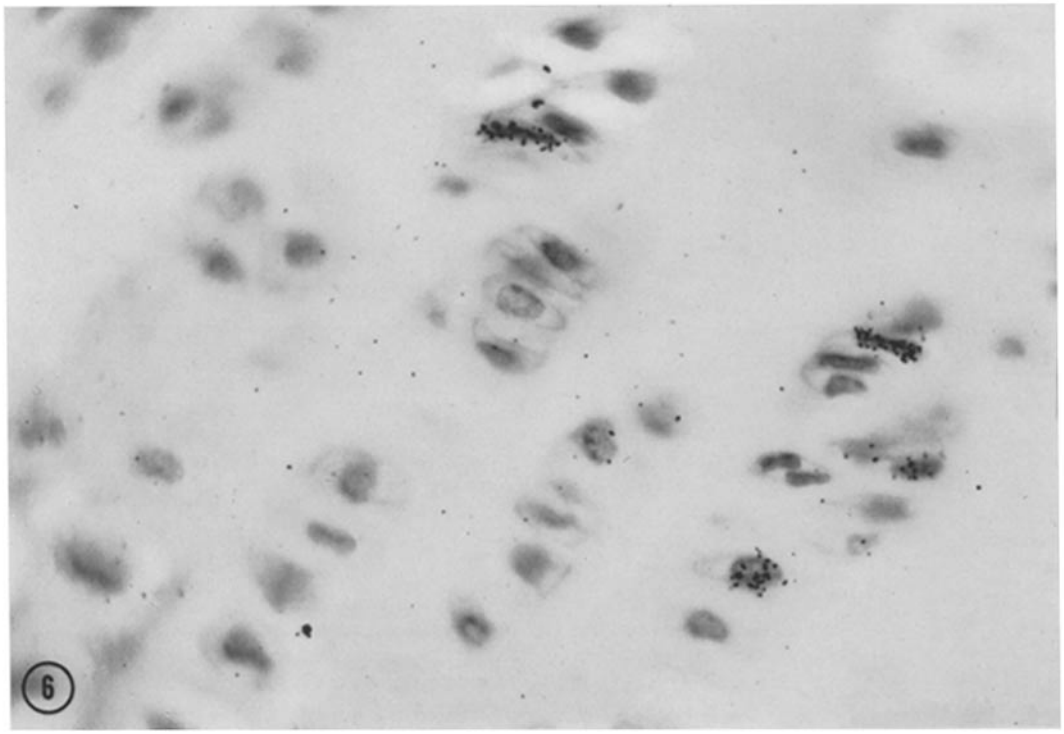
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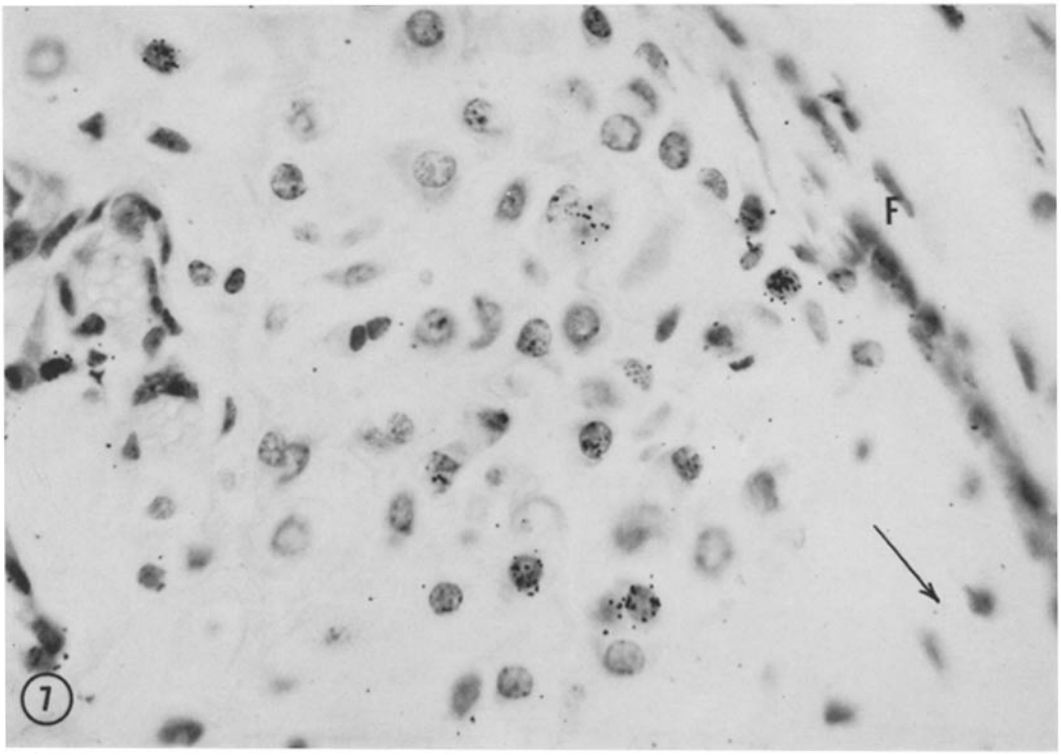
FIGURE 7

Autoradiograph prepared from the posterior aspect of the distal femoral epiphysis of a 5-week-old mouse. The animal was sacrificed 3 days after isotope administration. Note that the label now appears farther away from the perichondrial zone (arrow) and in well defined cartilage cells. *F* indicates the fibrous layer of the perichondrium. Harris hematoxylin stain. Oil immersion. × 410.

FIGURE 8

Autoradiograph of the posterior aspect of the distal femoral epiphysis of a 6-week-old mouse. The animal was sacrificed 4 days after isotope administration. Owing to successive divisions, the labeled cells which are located entirely within the epiphysis at this time show fewer grains. The arrow indicates the direction of the diaphysis, not seen in the photomicrograph. *F* represents the fibrous layer of the perichondrium. Harris hematoxylin stain. Oil immersion. × 410.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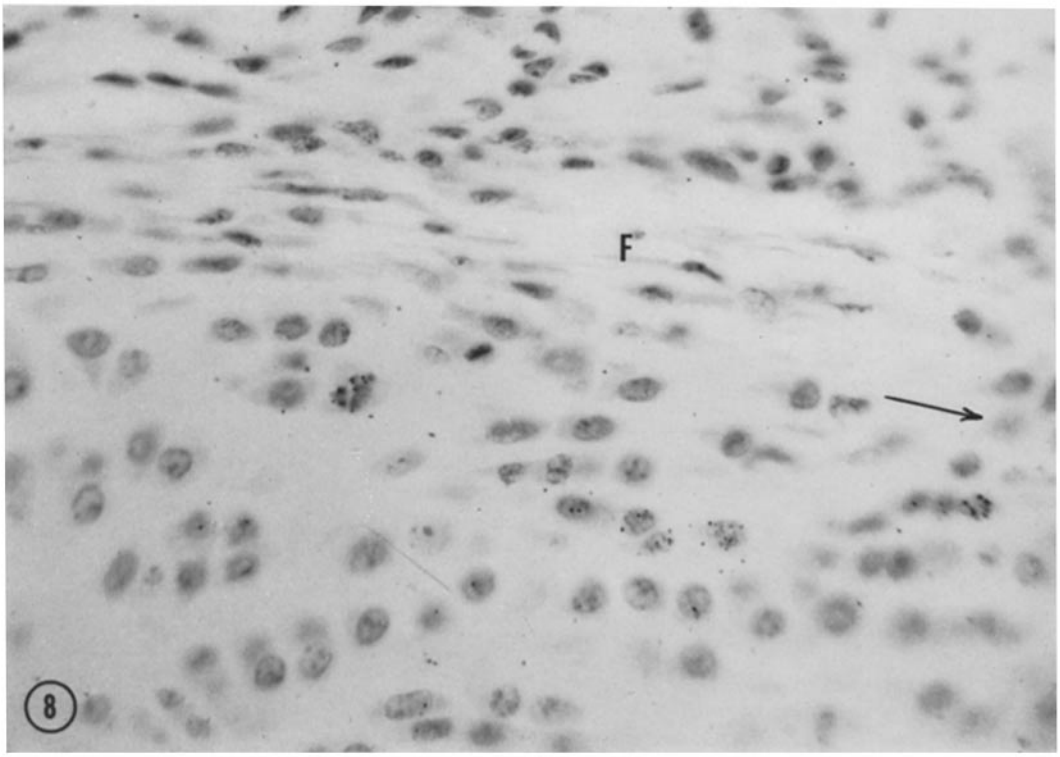
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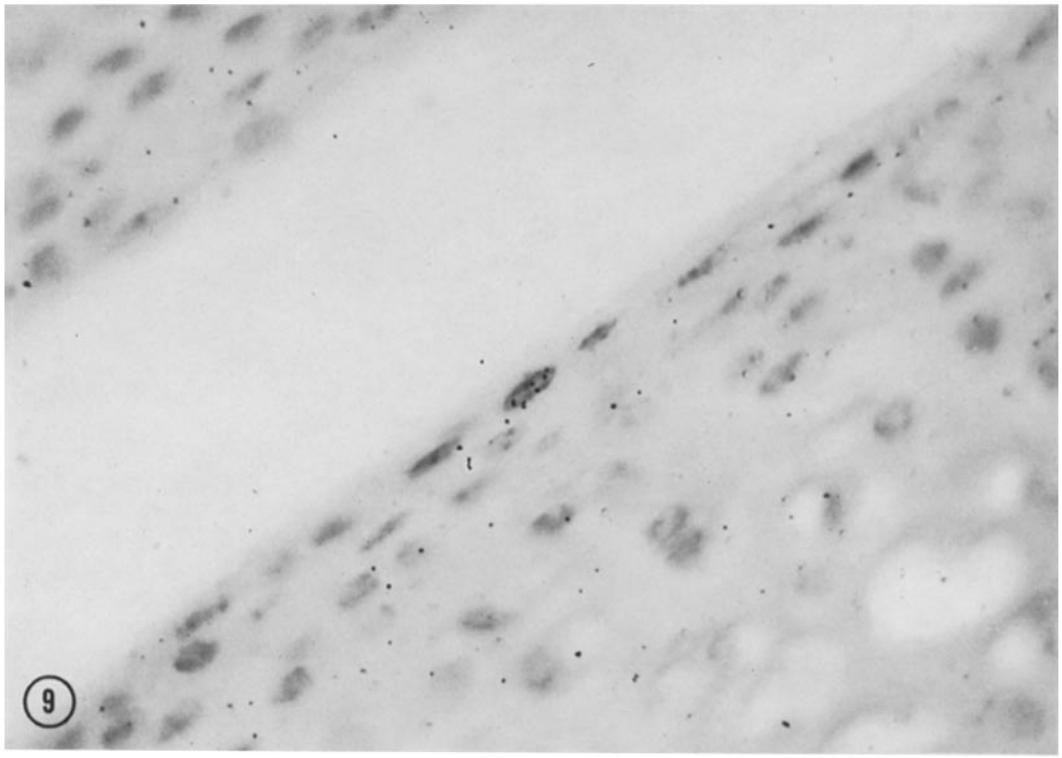
FIGURE 9

Autoradiograph of the articulating surface of the distal femoral epiphysis of a 5-week-old mouse. Labeled cells are rarely found at the articulating surfaces 1 hour after isotope administration. Harris hematoxylin stain. Oil immersion. × 676.

FIGURE 10

Autoradiograph similar to Fig. 9, prepared, however, 6 days after isotope administration. At this time the label is very weak and appears more frequently in cells at the articulating surface. Harris hematoxylin stain. Oil immersion. × 405.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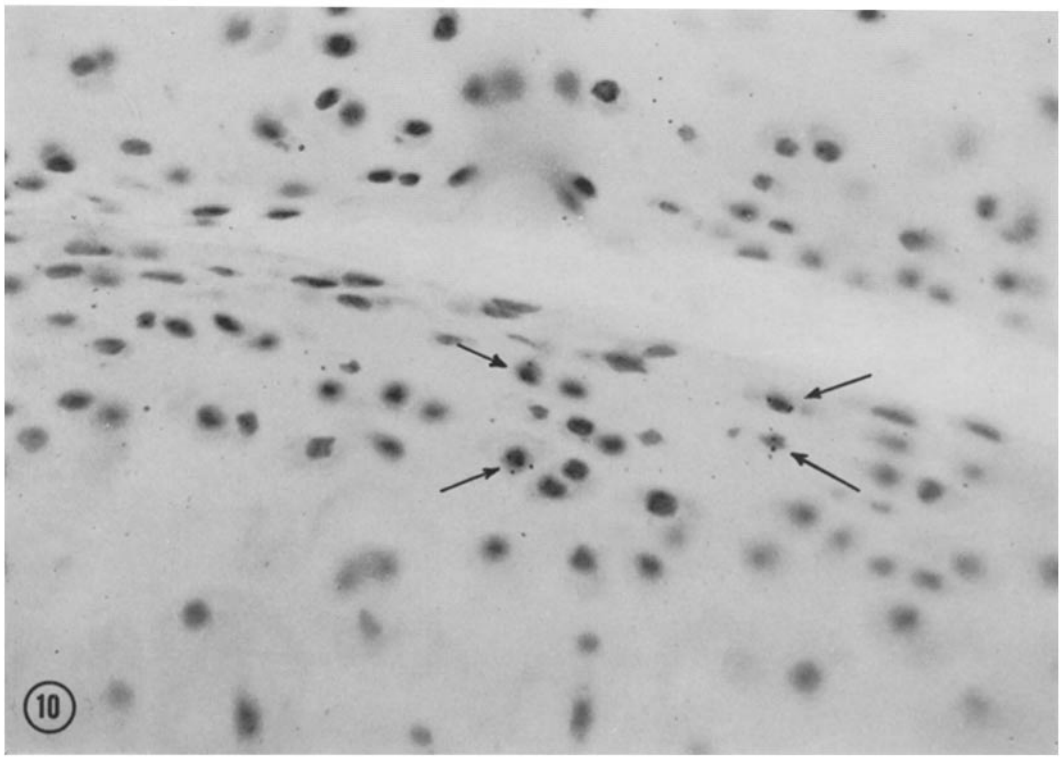
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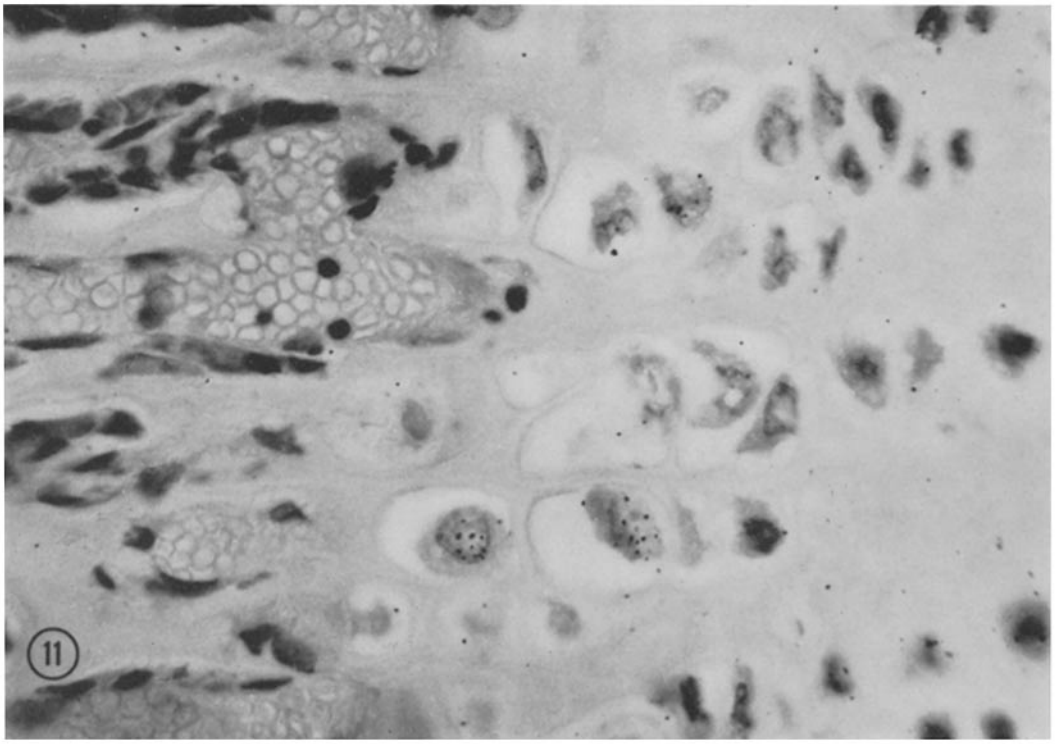

FIGURE 11

Autoradiograph of the distal femoral epiphyseal disk of a 5-week-old mouse, 4 days after isotope administration. Degenerating cartilage cells of the disk are seen labeled. Harris hematoxylin stain. Oil immersion. × 410.

FIGURE 12

Diagrammatic representation of the distal end of mouse femur. The arrows indicate directions of growth. Periosteal bone is indicated by the stippled area. Crosshatched areas indicate the perichondrial region which supplies cells to the epiphyseal disk and articulating surfaces. The major contribution of cells is made by the posterior aspect of the femur. The role of the periosteum in circumferential, longitudinal, and epiphyseal bone growth is shown.