CELL PROLIFERATION AND SPECIALIZATION DURING ENDOCHONDRAL OSTEOGENESIS IN YOUNG RATS

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ABSTRACT

Endochondral osteogenesis was studied autoradiographically in ribs and tibiae of 32 Long-Evans rats injected with 1 μc/gm H^3-thymidine at 6 days of age and sacrificed at intervals between 1 hour and 2 weeks later. Proliferation and specialization of bone cells were studied by analyses of (*a*) the percentage of mitoses which were labeled, (*b*) the percentage of labeled nuclei in bone cells, and (*c*) grain counts. The following conclusions were derived: The various types of bone cells represent different functional states of the same cell. Cell division is usually restricted to cells in the morphologically unspecialized "osteoprogenitor" state. Specialized bone cells arise by modulation of osteoprogenitor cells. The average cell generation time is shortest in the metaphysis, longest in the periosteum, and intermediate in the endosteum. The average duration of DNA synthesis is relatively constant (about 8 hours). With increasing length of generation time there is a slight increase in G_2 + mitosis, but the major change is a lengthening of G_1. After dividing, cells in the osteoprogenitor state may remain within the progenitor pool or undergo modulation of cell type, specializing as osteoblasts or becoming incorporated in osteoclasts.

INTRODUCTION

The usefulness of H^3-thymidine in autoradiographic analyses of cell proliferation and specialization has been firmly established. Incorporation of the nucleoside, which is a specific precursor of DNA, is restricted to cells undergoing DNA synthesis. The nuclear label is retained as long as these cells survive, except for dilution by subsequent mitosis. Consequently, the fate of labeled cells may be traced by examination of specimens at progressively longer intervals after injection.

The present study concerns the cells of bone in sites of endochondral osteogenesis in young rats. In this population of morphologically variable cells, thymidine uptake, and thus DNA synthesis and mitosis, is primarily restricted to "osteoprogenitor" cells, which are connective tissue cells of relatively unspecialized appearance (36). A brief report has been presented dealing with some aspects of cell proliferation in this material (35). It was found that in tibiae of young rats the average interval between cell divisions was shortest in the metaphysis, longest in the periosteum, and intermediate in the endosteum. These differences were due mainly to changes in the length of the "pre-DNA-synthetic phase" of interphase.

The present report extends the analysis to include the costochondral junction. In addition, the results of an investigation of the transformation of proliferating cells into specialized bone cells are presented.

MATERIALS AND METHODS

Thirty-two, 6-day-old Long-Evans rats were injected intraperitoneally with 0.1 ml of isotonic saline con-

taining 1 μc/gm body weight of H^3-thymidine (Schwarz Bioresearch, Inc., Mt. Vernon, New York, sp. act., 0.36 c/mM). All injections were made between 8 and 9 a.m. Animals were then sacrificed at intervals between 1 hour and 2 weeks later. Mothers were supplied with Purina laboratory chow (Ralston Purina Co., St. Louis, Missouri) and water *ad lib.*, supplemented by fresh lettuce once each week. Shortly after birth litter size was reduced to 8 animals.

At sacrifice the proximal half of one tibia and the costochondral junctions of three ribs were removed and fixed in Bouin-Hollande solution for 3 days. Decalcification was completed in 18.5 per cent aqueous versene adjusted to pH 7 with HCl. Tissues were double-embedded in nitrocellulose-paraffin, sectioned at 6 μ and prepared for autoradiography with Kodak NTB2 liquid emulsion. Exposure was for 2 weeks at 4°C under low humidity. Autoradiograms were developed in Dektol for 2 minutes at 17°C. Some were stained with periodic acid–Schiff (PAS) before autoradiography and hematoxylin after development. Others were stained with hematoxylin alone, after development.

Classification of Cells

Cell types within the bone series were categorized as follows:

Osteoprogenitor cells: Cells on or near the surface of bone or calcified cartilage with inconspicuous cytoplasm and generally vesicular, pale-staining nuclei, often oval or fusiform (Figs. 1, 2). This includes the spindle and reticulum cells of other authors, and the mesenchyme cells of Kember (19) and Young (35). The term "mesenchyme" implies a vast range of possible specializations of which only a few are documented below. Consequently, the more restrictive term "osteoprogenitor[1] cell" has been employed. Additional studies may reveal that the broader term is indeed applicable.

Osteoblasts: Cells lining the surface of bone or calcified cartilage with round or oval nucleus, prominent nucleolus, abundant basophilic cytoplasm, often with a juxtanuclear vacuole (Figs. 1, 2). A few cells intermediate in morphology between these two categories are seen. These were classed as either osteoblasts or osteoprogenitor cells according to the judgment of the observer.

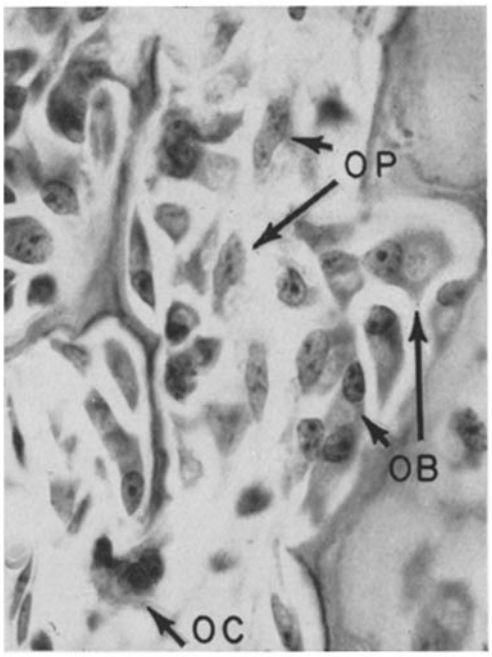

FIGURE 1

Proximal tibial metaphysis of a 6-day-old rat, showing osteoprogenitor cells (*OP*), osteoblasts (*OB*), and an osteoclast (*OC*). Hematoxylin and eosin. × 800.

Osteocytes: Cells contained within bone lacunae.

Osteoclasts: Multinuclear cells on or near the surface of bone or calcified cartilage (Fig. 1).

Methods of Analysis

Cell proliferation: A) The percentage of osteoprogenitor cells containing labeled nuclei was recorded separately for the metaphyseal, endosteal, and periosteal regions (excluding the perichondrial zone, *cf.* 28) of ribs and tibiae from the three rats sacrificed at 1 hour, to determine the proportion of cells engaged in DNA synthesis at the time of injection (Fig 3).

B) In order to follow these labeled cells through subsequent mitoses the percentage of labeled mitotic figures (all recognizable stages) was recorded in the metaphyseal region of ribs at all intervals. In animals sacrificed between 1 and 12 hours, this percentage was determined for the endosteum and periosteum as well. A comparable analysis of the tibia has been reported (35).

[1] The confusion which exists in regard to naming these cells is indicated by the discussion on terminology given by McLean and Urist (21). The term "osteoprogenitor" has the advantages of (*a*) clearly implying the progenitive function indicated in the data here reported, and (*b*) harmonizing with the nomenclature of the specialized cells of bone. With one exception (19), the need for clear, morphological description of cell categories has generally been overlooked in reports of H^3-thymidine autoradiographic analyses of bone.

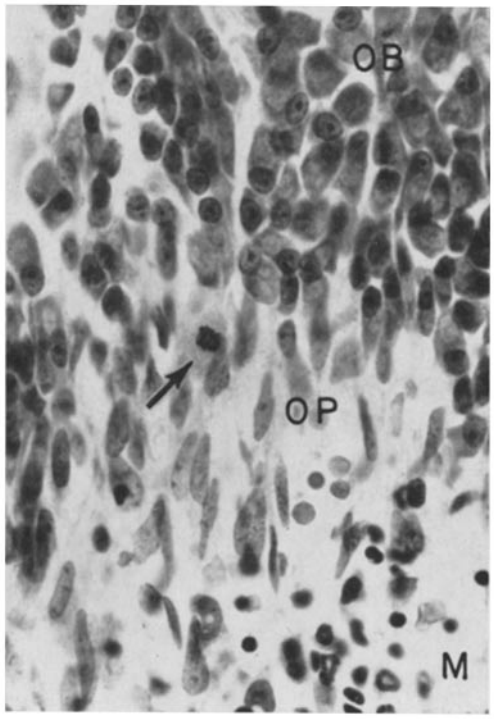

FIGURE 2

Tangential section through the rib endosteum of a 6-day-old rat, showing osteoblasts (*OB*), osteoprogenitor cells (*OP*), and bone marrow (*M*). Note mitotic osteoprogenitor cell (arrow). Hematoxylin and eosin. × 640.

Cell specialization: In order to record the subsequent specialization of initially labeled osteoprogenitor cells A) the percentage of labeled osteoblasts was computed separately for the endosteum, periosteum, and metaphysis at all ages in both ribs and tibiae.

B) In both bones at all intervals the percentage of osteoclasts containing one or more labeled nuclei was recorded, in this case without regard to location.

C) In thirteen selected animals sacrificed between 16 hours and 2 weeks after injection the percentage of osteoclast nuclei which were labeled was noted separately in each of the three regions of bone. The metaphyseal region was further subdivided into proximal, distal, and middle sectors (Fig. 3).

D) In sections from ribs and tibiae of fourteen rats sacrificed between 9 hours and 2 weeks the percentage of labeled osteoblast nuclei was determined separately for the proximal and distal metaphyseal zone (Fig. 3).

E) Counts of total reduced silver grains over randomly selected, labeled nuclei of osteoblasts, osteoclasts, and osteocytes were carried out separately for the metaphyseal, endosteal, and periosteal regions of tibiae from animals sacrificed at 1, 4, 7, and 14 days after injection. Nuclei overlain by fewer than 4 grains were not counted. Grain counts over the osteoprogenitor cells have been reported (35). Except as noted in the text, grains over 50 to 200 cells were counted in each regional cell category.

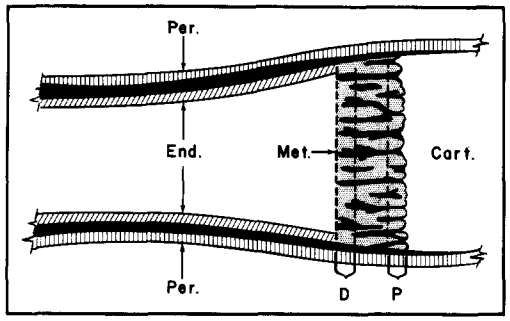

FIGURE 3

Diagram of a bone, indicating the regions analyzed in ribs and tibiae. *Per.*, periosteum (vertical lines; the perichondrial zone was not included); *End.*, endosteum (slanted lines); *Met.*, metaphysis (stippled). The metaphyseal region has been subdivided into distal (*D*), proximal (*P*), and middle segments. The distal segment includes the area of trabecular resorption and the transition zone. The proximal segment includes the area of calcified cartilage resorption and the immediately adjacent portion of the trabeculae (*cf.* Fig. 8). Bone and calcified cartilage are indicated in black. *Cart.*, cartilage.

RESULTS

Cell Proliferation

In corroboration of previous reports, it was found that with few exceptions (noted below), only osteoprogenitor cells incorporated H^3-thymidine (Figs. 4, 5). The frequency with which these cells were initially labeled varied regionally (Table I). In general it was highest in the metaphysis, lowest in the periosteum, and intermediate in the endosteum.

In all three regions of both bones variations in the percentage of labeled mitotic figures between 1 and 12 hours followed a similar trend, comprising an initial rapid rise from zero to a temporary plateau at about 100 per cent, followed by a more gradual decline, which did not, however, return to zero (Table II). Labeled mitotic figures were not observed at 1 hour. At 1.5 hours a few weakly

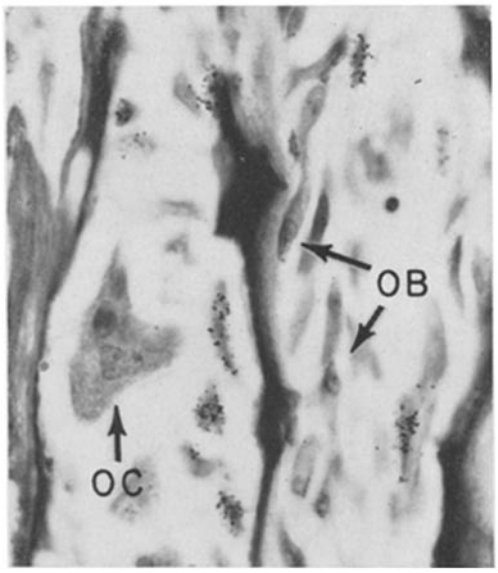

FIGURE 4

Tibial metaphysis of a 6-day-old rat sacrificed 1 hour after injection of H^3-thymidine. Incorporation of thymidine is restricted to osteoprogenitor cells. Osteoblasts (*OB*) and osteoclasts (*OC*) are unlabeled. Autoradiogram, PAS-hematoxylin. × 800.

TABLE I

*Percentage of Labeled Osteoprogenitor Cells in Different Regions of Ribs and Tibiae 1 Hour after Injection of H^3-Thymidine**

Bone	Metaphysis	Endosteum	Periosteum
Tibia	22‡	14‡	7‡
	23	13	7
	22	12	5
Rib	24	15	2
	26	14	6
	24	12	4

* Each percentage = 5,000 or more cells counted.
‡ Taken from Young (35).

labeled mitoses were found in the metaphysis and endosteum of ribs and tibiae. Radioactive dividing cells were first observed periosteally at 2 hours in both bones. The maximum labeling, near 100 per cent, was attained by 3 hours in the metaphysis but not until 4 hours in the endosteum and periosteum. The subsequent drop in labeling frequency began slightly earlier in the metaphysis and endosteum (8 to 9 hours) than in the periosteum (9 to 10 hours). The interval between the first attainment of 50 per cent labeled mitoses and the later drop to this level occupied about 8 hours in all regions of both bones. The significance of this will be noted later.

In summary, the rise and fall in the percentage of labeled mitotic figures followed a similar course in both bones during the first 12 hours but was regionally slightly displaced in time, the periosteum lagging about ¾ hour behind the metaphysis with the endosteum occupying an intermediate position.

TABLE II

*Percentage of Labeled Mitotic Figures in Different Regions of Ribs and Tibiae between 1 and 12 Hours after Injection of H^3-Thymidine**

Hours	Metaphysis‡		Endosteum§		Periosteum§	
	Rib	Tibia	Rib	Tibia	Rib	Tibia
1	0	0	0	0	0	0
1.5	11	7	4	6	0	0
2	62	50	30	28	12	20
2.5	78	74	57	54	22	38
3	96	98	87	92	86	85
4	96	96	95	97	92	96
5.5	99	99	100	98	98	98
7	97	95	94	98	100	96
8	93	93	92	94	94	96
9	93	90	92	92	98	98
10	52	56	60	57	84	82
12	14	11	23	19	30	22

* Figures for tibia taken from Young (35) with addition of animal at 5.5 hours.
‡ Each percentage = 100 or more mitoses counted.
§ Each percentage = 50 or more mitoses counted.

In the metaphysis a broad, attenuated second rise in per cent labeled mitoses appeared at about 36 to 40 hours in both bones (Table III). Thereafter, the percentage stabilized at slightly over 20 per cent, then declined again 1 week after injection. By 2 weeks no labeled mitoses could be detected in the metaphysis of either bone.

Cell Specialization

The percentage of osteoblasts containing labeled nuclei (Figs. 6, 7) in the three regions of ribs and tibiae is given in Table IV. At early intervals a very small percentage of osteoblasts was usually labeled in the metaphysis and endosteum

but not in the periosteum, where reactive osteoblasts were observed consistently only after 20 hours (tibia) or 36 hours (rib). In the metaphysis and endosteum the percentage began to increase between 9 and 16 hours, reaching a peak earliest in the rib metaphysis (24 to 28 hours), later in the tibial metaphysis (36 hours), and later still (about 2 days) in the other regions of both bones. The labeling frequency became stabilized at levels which differed markedly among the three regions of these bones. In each region the level attained, but did not appreciably exceed, on the average, the percentage of osteoprogenitor cells initially labeled in each region (*cf.* Table I). In the metaphysis, and to a lesser degree in the endosteum, the percentage of labeled osteoblasts decreased in the older animals.

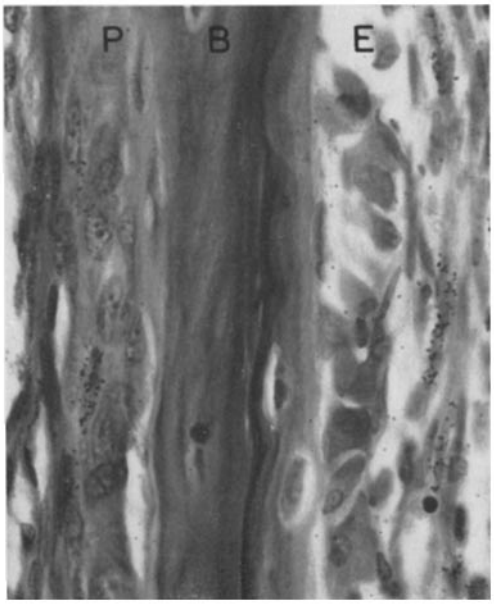

FIGURE 5

Rib shaft of a 6-day-old rat sacrificed 1 hour after injection of H^3-thymidine. Only osteoprogenitor cells of the periosteum (*P*) and endosteum (*E*) are labeled. *B*, bone. Autoradiogram, PAS-hematoxylin. × 640.

When the metaphyseal region of ribs and tibiae was subdivided into proximal and distal segments (Fig. 3) and the percentage of labeled osteoblasts recounted at selected intervals (Table V), it was observed that in both bones the percentage rose more steeply in the proximal compared to the distal zone. By 2 days after injection, however, both regions were approximately equally labeled and remained so until about 1 week. Therefore the percentage dropped.

Table VI gives the percentage of osteoclasts which contained at least one labeled nucleus in ribs and tibiae at all intervals studied. Labeled nuclei were not found in these cells until 9 hours. After 9 hours the percentage rose steeply, leveled off at about 40 hours, and finally decreased after 122 hours.

TABLE III

Percentage of Labeled Mitotic Figures in the Metaphysis of Ribs and Tibiae between 16 and 336 Hours after Injection of H^3-Thymidine

Hours	Percentage of labeled mitoses*	
	Rib	Tibia‡
16	19	15
20	15	20
24	15	17
28	32	28
33	28	30
36	38	37
40	37	38
45	34	30
50	25	29
58	20	26
65	20	19
72	18	21
80	21	21
88	22	22
96	20	20
122	27	23
168	12	17
336	0	0

* Each percentage = 100 or more mitoses counted.
‡ Taken from Young (35).

In Table VII the percentage of labeled osteoclast *nuclei* (according to region) is presented (Fig. 6). Within the metaphysis and periosteum the maximum labeling frequency attained was again similar to the original percentage of labeled osteoprogenitor cells (*cf.* Table I), although in the metaphysis it was reached somewhat later and at a slightly lower level than the maximum of labeled osteoblasts. Labeling of osteoclast nuclei endosteally was distinctive in that it remained generally well below the percentage of labeled osteoblasts in the same region (*cf.* Table IV). In the periosteum

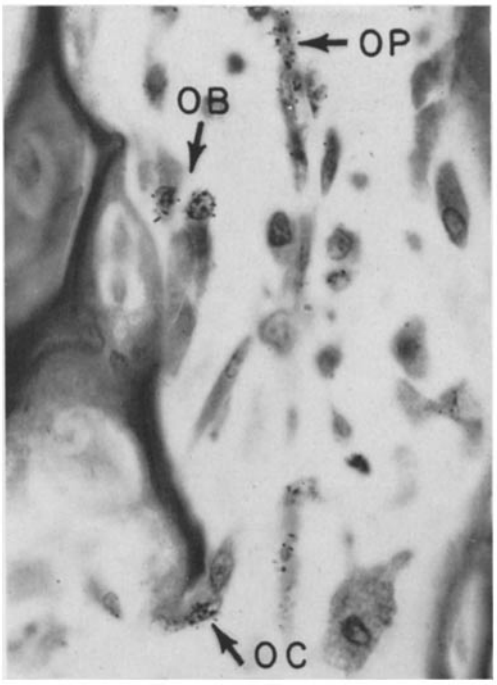

FIGURE 6

Tibial metaphysis of a 7-day-old rat sacrificed 33 hours after injection of H^3-thymidine. Labeled nuclei occur in an osteoprogenitor cell (*OP*), osteoblasts (*OB*), and an osteoclast (*OC*). Autoradiogram, PAS-hematoxylin. × 800.

of ribs, labeled osteoclasts appeared before labeled osteoblasts were seen (*cf.* Table IV).

A metaphyseal proximal-distal gradient in labeling rate was also observed in osteoclasts. The percentage of labeled nuclei rose more steeply nearer the cartilage, compared to the distal zone bordering on the marrow. However, by about 2 days the percentage was comparable in both regions. In the older animals (122 to 336 hours) the labeled percentage declined in all metaphyseal zones but was not altered markedly in the endosteum and periosteum.

The first labeled osteocyte was observed 50 hours after injection. Thereafter, labeled osteocytes were always observed in the shaft and metaphysis (Fig. 7) except at 2 weeks, by which time no cells were labeled above background in the metaphysis at the exposures used.

Grain counts over nuclei of osteoprogenitor cells (35), osteoblasts, and osteoclasts were similar 1 day after injection in tibiae. Most counts fell between 20 and 50 grains with a peak at roughly 30 to 40 grains. At 4 days the suggestion of a slight drop in grain count over osteoprogenitor cells in the metaphysis and endosteum relative to the periosteum began to be manifest (35). By 7 days it was apparent that decrease of labeling intensity in specialized bone cells lagged behind that of the osteoprogenitor population. The progressive dilution of radioactivity in general was most rapid in the metaphysis, slowest in the periosteum, and intermediate in the endosteum. For example, 7

TABLE IV

Percentage of Labeled Osteoblasts in Different Regions of Ribs and Tibiae between 1 and 336 Hours after Injection of H^3-Thymidine

Hours after injection	Metaphysis*		Endosteum‡		Periosteum‡	
	Rib	Tibia	Rib	Tibia	Rib	Tibia
1§	2	1	1	1	0	0
1.5	3	2	1	0	0	0
2	2	2	0	0	0	0
2.5	3	2	0	1	0	0
3	1	1	0	1	0	0
4	2	2	1	0	0	0
5.5	2	2	1	2	0	0
7	2	1	1	1	0	0
8	5	1	0	0	0	0
9	7	2	0	1	0	0
10	10	4	2	1	0	0
12	15	2	1	1	0	1
16	11	5	2	2	0	0
20	16	7	1	4	0	1
24	21	9	2	8	0	1
28	24	10	2	8	1	4
33	23	14	5	8	0	2
36	21	22	8	14	1	3
40	24	24	6	9	1	3
45	25	23	7	11	2	6
50	25	22	12	12	4	6
58	27	26	12	14	4	7
65	21	23	15	12	5	6
72	22	22	11	14	5	4
80	23	25	13	13	5	7
88	20	23	14	12	4	8
96	25	24	17	13	3	8
122	14	19	19	16	6	7
168	6	12	17	14	4	8
336	0	0	6	6	6	8

* Each percentage = 500 or more osteoblasts counted.

‡ Each percentage = 300 or more osteoblasts counted.

§ Average of three animals.

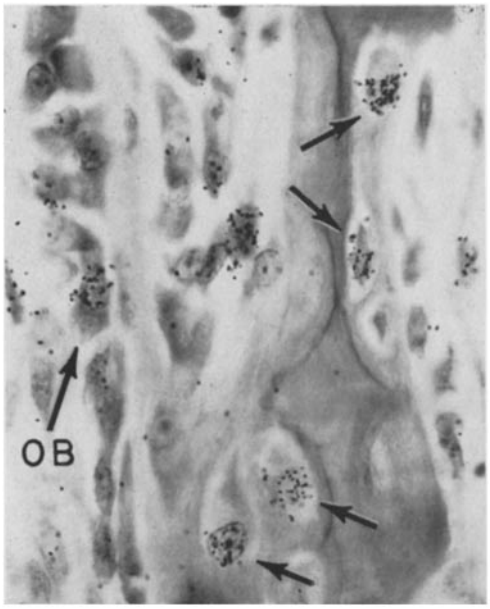

FIGURE 7

Tibial metaphysis of a 10-day-old rat sacrificed 96 hours after injection of H^3-thymidine. Several osteocytes (arrows) and an osteoblast (*OB*) are labeled. Autoradiogram, PAS-hematoxylin. × 800.

TABLE V

*Percentage of Labeled Osteoblasts in Proximal and Distal Regions of the Metaphysis of Ribs and Tibiae at Various Intervals after Injection of H^3-Thymidine**

Hours after injection	Rib		Tibia	
	Proximal	Distal	Proximal	Distal
9	12	4	2	1
16	20	7	6	2
20	22	8	9	5
24	24	15	13	8
33	25	18	14	11
40	25	14	23	20
45	26	23	25	21
50	26	25	22	18
58	27	27	27	25
72	22	25	23	23
96	23	25	25	27
122	11	19	21	23
168	4	7	7	16
336	0	0	0	0

* Each percentage = 200 or more osteoblasts counted. *Cf.* Fig. 3.

TABLE VI

*Percentage of Osteoclasts Containing One or More Labeled Nuclei in All Regions of Ribs and Tibiae between 1 and 336 Hours after Injection of H^3-Thymidine**

Hours after injection	Rib	Tibia	Hours after injection	Rib	Tibia
1	0	0	28	19	12
1.5	0	0	33	22	19
2	0	0	36	29	27
2.5	0	0	40	34	34
3	0	0	45	37	46
4	0	0	50	44	35
5.5	0	0	58	38	36
7	0	0	65	37	25
8	0	0	72	32	21
9	1	3	80	39	27
10	1	1	88	37	31
12	4	3	96	32	30
16	8	6	122	39	35
20	8	9	168	24	17
24	13	10	336	11	4

* Each percentage = 100 or more osteoclasts counted.

days after injection as few as 4 to 9 grains were observed over 70, 50, and 30 per cent of labeled osteoprogenitor cells in the metaphysis, endosteum, and periosteum, respectively. Specialized bone cells in general showed these same regional differences, except that osteocytes revealed a broader range of labeling, including the most heavily labeled cells observed (Fig. 7).

At 14 days in the metaphysis no nuclei of any cell type were significantly labeled (4 grains or more). In the endosteum almost 90 per cent of labeled osteoprogenitor cell nuclei were covered by only 4 to 9 grains. Sixty-five per cent of labeled osteoblasts and most labeled osteoclast nuclei (7 out of 13 observed) also fell within this group. In the periosteum only 50 per cent of osteoprogenitor cells and 25 per cent of osteoblasts, but most osteoclasts (6 out of the 10 observed), were covered by as little as 4 to 9 grains. In the shaft about 20 per cent of labeled osteocytes were overlain by 4 to 9 grains, the rest being more intensely labeled.

DISCUSSION

Analysis of H^3-thymidine autoradiograms is based on a number of assumptions, including the fol-

lowing: H^3-thymidine is a specific precursor of DNA, taken up only by cells undergoing DNA systhesis. There is no subsequent turn-over of DNA. Injected H^3-thymidine is rapidly removed from the blood stream and utilized or quickly degraded. Intranuclear irradiation damage is insignificant at 1 μc/gm over relatively short periods. Discussion of the validity of these and other premises is given elsewhere (1, 7, 9, 15, 16, 18, 22). There is some indication that the injection of thymidine itself may influence the proliferative cycle in adult mice, perhaps by shortening the DNA synthetic period (11). Other possible sources of error include mistaken classification of cells, and the inherent risk of estimating temporal trends by comparison of different animals.

TABLE VII

*Percentage of Labeled Nuclei in Osteoclasts in Different Regions of Ribs and Tibiae between 16 and 336 Hours after Injection of H^3-Thymidine**

Hours after injection	Metaphysis						Endosteum		Periosteum	
	Rib			Tibia			Rib	Tibia	Rib	Tibia
	Prox.‡	Total‖	Dist.§	Prox.‡	Total‖	Dist.§	Total	Total	Total	Total
16	6	4	3	5	3	1	0	0	4	0
20	9	7	5	7	5	4	0	0	4	0
24	15	12	7	14	9	3	0	2	1	0
33	19	13	5	18	11	2	0	1	7	2
40	16	12	9	16	10	7	1	1	5	2
45	19	20	20	24	22	21	0	2	5	6
50	25	22	21	21	20	18	5	1	4	3
58	20	19	18	20	21	21	3	4	8	9
72	15	16	17	16	16	15	3	2	4	4
96	18	22	22	16	17	17	3	5	7	6
122	13	14	15	18	17	17	10	13	7	6
168	6	8	9	10	10	11	6	4	8	4
336	0	0	0	0	0	0	7	5	4	2

* Each percentage = 100 or more nuclei counted, except for the tibial periosteum at 16, 20, and 24 hours where less than 25 osteoclast nuclei were observed.
‡ Prox. = proximal zone of metaphysis (Fig. 3).
§ Dist. = distal zone of metaphysis (Fig. 3).
‖ Total includes middle zone of metaphysis (Fig. 3).

Cell Proliferation

In a proliferating, non-synchronous cell population, only those cells undergoing DNA synthesis at the time of H^3-thymidine injection will become labeled. The interval between injection and earliest appearance of labeled mitoses represents the minimum interval between completion of DNA synthesis and onset of histologically recognizable mitosis (the minimum G_2 period). The time between injection and attainment of 100 per cent labeled dividing cells provides an estimate of the maximum length of G_2 + mitosis (M), since during this interval the unlabeled cells in G_2 or M pass into the succeeding phase (G_1). Ultimately, unlabeled mitotic cells (presumably in G_1 at the time of injection) begin to appear again in increasing numbers. The average duration of DNA synthesis (S) may be estimated from the interval between the initial attainment of 50 per cent labeled mitoses and the subsequent drop to 50 per cent after the intervening period at about 100 per cent labeling (6, 24, 25).

Assuming random distribution of phase, the percentage of cells undergoing DNA synthesis at the time of injection represents the percentage of the total proliferative cycle spent in the S phase. If the average duration of S is known, the average generation time (T) may be computed by

$$T = \frac{\text{DNA synthetic time}}{\text{Per cent in DNA synthesis}}$$

assuming that A) the total number of cells in the dividing population is relatively stable (with half the daughter cells being removed to non-proliferative compartments), and B) all cells assigned to the dividing population are actually proliferating.[2]

In the present material DNA synthesis was primarily restricted to osteoprogenitor cells. No labeled mitoses were observed at 1 hour, but at 1.5 hours a small percentage was radioactive in the metaphysis and endosteum of ribs and tibiae. In these sites, then, the minimum G_2 period was between 1 and 1.5 hours in duration. In the periosteum of both bones it was slightly longer—between 1.5 and 2 hours.

The percentage of labeled dividing cells rose steeply in both bones, so that effectively all were labeled by 3 hours in the metaphysis and by 4 hours in the endosteum and periosteum. Therefore, the maximum total duration of $G_2 + M$ was about 3 hours metaphyseally and about 4 hours in the other two regions. From Table II it can be estimated that the average duration of DNA synthesis (interval between initial and subsequent attainment of 50 per cent labeled mitoses) was approximately 8 hours in all regions of both bones.

Based on this 8-hour S period and the average percentage of cells in S derived from the three animals sacrificed at 1 hour, the average regional generation times are estimated to be about 36, 62, and 133 hours for the metaphysis, endosteum, and periosteum of tibiae and 32, 57, 200 hours for the three respective regions of ribs.

Grain counts over labeled osteoprogenitor cells in tibiae (35) support the finding that generation time was shortest metaphyseally, longest periosteally, and intermediate endosteally. This is important, since a low initial incidence of labeling (*e.g.*, periosteally) might mean A) a long generation time or B) a small progenitor population. If B) were correct, then repeated mitosis among the small population would lead to rapid dilution. If A) exists, dilution should be slow (as was observed). In general, dilution in each region was indicative of a single population. There was, nevertheless, variability in T within these regions, as indicated below.

The slight regional variations (1 hour or less) in $G_2 + M$ are consistent with the variations in generation time (*cf.* 5, 35) but are inadequate to account for the large variations in T. Since S is relatively constant, variability in T must therefore be due mainly to changes in length of the G_1 period, a finding consistent with observations on other cell systems. It has been reported (25) that all stages of the progenitor cycle (particularly G_1) may increase in certain slowly dividing cell populations.

Periodic analysis of metaphyseal mitoses in ribs and tibiae following the first division of labeled cells revealed a slight second rise in labeling frequency at 36 to 40 hours after injection, which probably represents the second division of an appreciable portion of initially labeled cells. This slight increase, roughly 31 to 35 hours after the first peak, tends to corroborate the predicted values for T, since it occurs at a time consistent with the estimated generation time in this region.

The slope of the initial rise from zero to approximately 100 per cent labeled mitoses reflects in part the variability of $G_2 + M$. The more gradual decline towards the base line includes the additional variability of S. The highly attenuated second peak results from the combined variabilities of two passages through S, G_2, and M, and the intervening G_1 period. After the first rise the percentage of labeled mitoses did not drop to zero, suggesting that some labeled cells entered mitosis for a second time before the last of the initially labeled cells had completed their first division.

After two generations the percentage of labeled mitoses became stabilized at a level comparable to the initial percentage of labeled osteoprogenitor cells in the metaphysis. Evidently, randomization of phase among labeled cells proceeded until these cells were equally distributed throughout the cycle. Obviously, very few cells will have generation times comparable to the predicted value, which gives only an approximation of the general rate of cell division in this material.

Cell Specialization

In the metaphysis and endosteum of both bones as few as 2 per cent or less of osteoblasts were labeled at 1 hour. The significant numbers of labeled osteoblasts observed in all regions at later intervals indicate that these cells must have been

[2] According to Kember (19), only 50 per cent of metaphyseal mesenchyme (osteoprogenitor) cells were labeled after 3 days of H^3-thymidine injections (spaced at 6 hour intervals) in 6- to 8-week-old rats. However, at this age generation time may be well over 3 days.

mainly derived from osteoprogenitor cells, the only cell type appreciably labeled at early intervals.

In each region the labeling frequency of osteoblasts became stabilized at a level comparable to the initial percentage of labeled osteoprogenitor cells. This suggests that most if not all osteoprogenitor cells were actually in the progenitor pool and equally capable of specializing as osteoblasts. The rate at which these osteoblasts came into labeling equilibrium with the osteoprogenitor cells in the metaphysis suggests a turn-over there of less than 2 days in these rats, which is consistent with turn-over of the metaphyseal trabeculae themselves, observed in H^3-glycine autoradiograms (Young, unpublished). Regional differences in the rate of appearance of labeled osteoblasts probably reflect variations in the rate of turn-over.

Turn-over of these cells in also reflected in the drop of per cent labeled osteoblasts first observed at 122 hours metaphyseally and 336 hours endosteally but not found periosteally. New osteoblasts are derived from the dividing, osteoprogenitor pool, in which dilution of radioactivity beyond detectable limits (at the exposures used)[3] varies regionally according to regional variations in generation time.

The progressive appearance of labeled nuclei in osteoclasts after 9 hours demonstrates that these cells grow by fusion of precursor cells. The percentage of labeled nuclei in metaphyseal and periosteal osteoclasts in both bones rose to a level comparable to the percentage of initially reactive osteoprogenitor cells in those zones. Evidently osteoprogenitor cells are equally competent to form both osteoblasts and osteoclasts.

Endosteally, labeled osteoclast nuclei appeared relatively late, and there was no definite tendency for the labeling frequency to rise appreciably. Perhaps growth of osteoclasts by fusion of nucleated elements is slow endosteally, and dilution of radioactivity by mitosis of labeled precursors may have progressed to the point where it was undetectable before equilibration of labeled osteoprogenitor and osteoclast nuclei occurred.

Cell specialization decreased the rate at which dilution occurred, since the specialized bone cells were generally incapable of mitosis. As the labeled osteoprogenitor cells continued to divide, increasingly diluting their radioactivity, each generation gave rise to specialized cells of correspondingly reduced labeling intensity. Thus, at later intervals, the labeling intensity over specialized cells was generally greater than that over osteoprogenitor cells, and covered a wider *range* of intensity, particularly in the case of osteocytes. The retention of heavy labeling in some of these cells suggests that osteocytes survive intact until reached by resorption. In contrast, heavily labeled nuclei were *not* long retained in osteoblasts and osteoclasts. Evidently these represent rather transient specialized states.

H^3-thymidine autoradiography has established that osteoclasts are formed by coalescence of uninucleated precursors. However, previous studies have led to the disparate conclusions that the precursors are osteoblasts (26, 27, 29), osteocytes (22), mesenchyme (osteoprogenitor) cells (19), or simply cells of undetermined nature (2, 17).

Nevertheless, most studies, including the present one, have consistently shown that the only cell type initially labeled in numbers adequate to account for the frequency of subsequent osteoclast labeling is the osteoprogenitor cell.

The problem resolves itself, then, into the question of whether there must be an intermediate stage between osteoprogenitor and osteoclast. The current material demonstrates that an intermediate stage is not necessary.

Osteoclasts associated with resorption of calcified cartilage in the proximal metaphyseal zone show a rapid rate of incorporation of labeled nuclei. There are very few osteoblasts in this zone in these animals, but abundant osteoprogenitor cells are present, and many become initially labeled. Secondly, labeled periosteal osteoclasts were observed in ribs several hours before labeled osteoblasts were seen in this region. The osteoblast stage, therefore, is not a prerequisite for incorporation of osteoprogenitor cells in osteoclasts.

Osteoblasts, however, may return to the osteoprogenitor state (14, 20, 36) from which they could thereafter presumably become incorporated

[3] Prolonged exposure of autoradiograms reveals activity in labeled nuclei insufficiently radioactive to produce an autoradiographic reaction in 2 weeks. Preliminary results of such a study indicate a higher than anticipated labeling frequency at low intensity in many cell types in some of the older animals, suggesting the possibility of reutilization of H^3-thymidine derived from labeled cells which had undergone cytolysis (30).

into osteoclasts. The *direct* incorporation of osteoblasts into osteoclasts seems less likely. These two highly specialized cell types differ metabolically and structurally in many ways (4, 8, 31, 34, 36) so that an intermediate stage of "despecialization" would probably be necessary. It appears to be a general rule that in higher forms transformation of cell type occurs between relatively unspecialized types, or from relatively unspecialized to specialized (10, 12).

The osteocyte stage is similarly an unnecessary intermediate between osteoprogenitor and osteoclast, since labeled osteoclasts are observed before labeled osteocytes become evident. Nevertheless, the fate of osteocytes freed by resorption is unknown, and they probably remain capable of transformation into the other cells of bone.

It has often been stated that the life span of osteoclasts is short, *e.g.*, 2 days (13). The concept of "life span," however, implies that the cells are first formed, and later destroyed, each in a single, brief operation (which may encompass hundreds of nuclei!). H^3-thymidine autoradiography, however, indicates that osteoclasts *progressively* incorporate nuclei. The gradual disappearance of heavily labeled osteoclast nuclei suggests that an associated progressive loss of nuclei also occurs. Consequently, osteoclasts may undergo a continual *turn-over* of nucleated *components*. The life span of the entire multinucleated cell might considerably exceed the duration within it of an individual nucleated element.

The fate of transitory osteoclast nuclei (and associated cytoplasm) remains unclear. They may disintegrate either within the osteoclast or after becoming detached from it. Or, having become detached, they may re-enter the dividing progenitor pool. Since cell death has repeatedly been reported to be negligible in growing bone, the second alternative seems more likely (26, 27).

The preceding analysis indicates the following course of events in the metaphysis (Fig. 8). At the zone of cartilage erosion, osteoclasts migrate proximally along with the receding cartilage front (19), participating in the resorption of calcified cartilage while undergoing turn-over of their component nuclei (and associated cytoplasm). Just distal to this resorptive area, osteoblasts are continually formed by specialization of osteoprogenitor cells (and perhaps to a very limited extent by self-reproduction). The osteoprogenitor cells also migrate proximally (19). The osteoblasts remain stationary, relative to the adjacent calcified cartilage, and deposit a thin layer of bone matrix on its surface. The distal ends of the trabeculae are resorbed by osteoclasts so that trabecular length remains approximately constant. This distal resorption front thereby continually encroaches on osteoblasts in the "transition zone." These cells may be incorporated into the osteoclasts after despecialization. The relatively late appearance of labeled nuclei in distal osteoclasts is at least compatible with this view. Of course, the osteoclasts would have to continually shed nuclei and cytoplasm to avoid increase in size. If the osteoblasts are *not* incorpo-

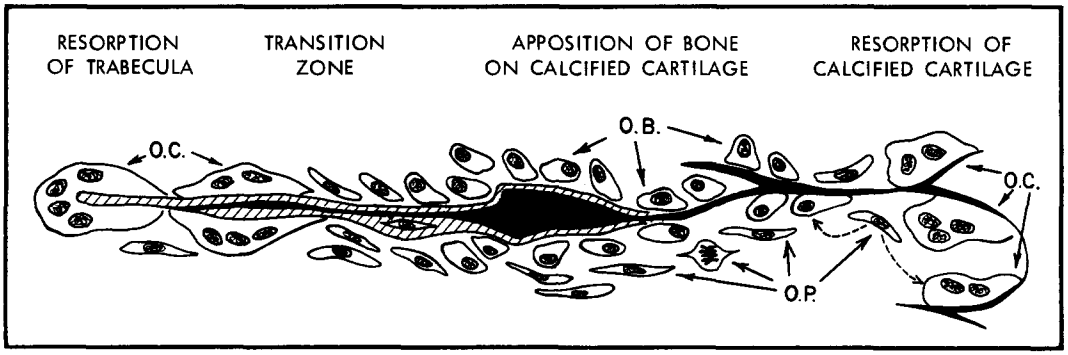

FIGURE 8

Diagram of a metaphyseal trabecula. The proximal segment is on the right (*cf.* Fig. 3). *O.P.*, osteoprogenitor cells (one of which is undergoing mitosis); *O.B.*, osteoblasts; *O.C.*, osteoclasts. Bone is depicted by slanted lines, calcified cartilage in black. The dashed arrows (right) indicate the origin of osteoblasts and osteoclasts from osteoprogenitor cells.

rated in the osteoclasts, they may detach themselves from the trabeculae and rejoin the progenitor population.

Osteoprogenitor cells are transformed into specialized bone cells by *modulation*, which yields a *temporary* cellular organization, persisting only as long as the respective conditioning environment is present. This contrasts with *differentiation* in which a *permanent* residue of the response to a particular environment has become "fixed" in the cell so that it can continue itself in the absence of the organizing environment (32). Many studies, including the present one, illustrate that the cells of bone are capable of shifting reversibly among a limited repertory of tasks (*e.g.*, 3, 23, 33, 36). These shifts involve a preferential utilization of part of the total genetic information stored in nuclear DNA. Due to differentiation, only a limited part of the total information remains available. Within these limits, modulation permits temporary, selective utilization from among the remaining alternatives (36).

During what phase of the progenitor cycle does specialization of osteoprogenitor cells occur? In the case of osteoblasts in the periosteum (where no labeled osteoblasts occurred initially in this material), and with regard to osteoclasts, the failure of initial labeling precludes modulation during the DNA synthetic phase. No mitoses were seen in these osteoblasts or osteoclasts, so that modulation did not occur during G_2 or M. Consequently, modulation probably occurs during the G_1 phase.

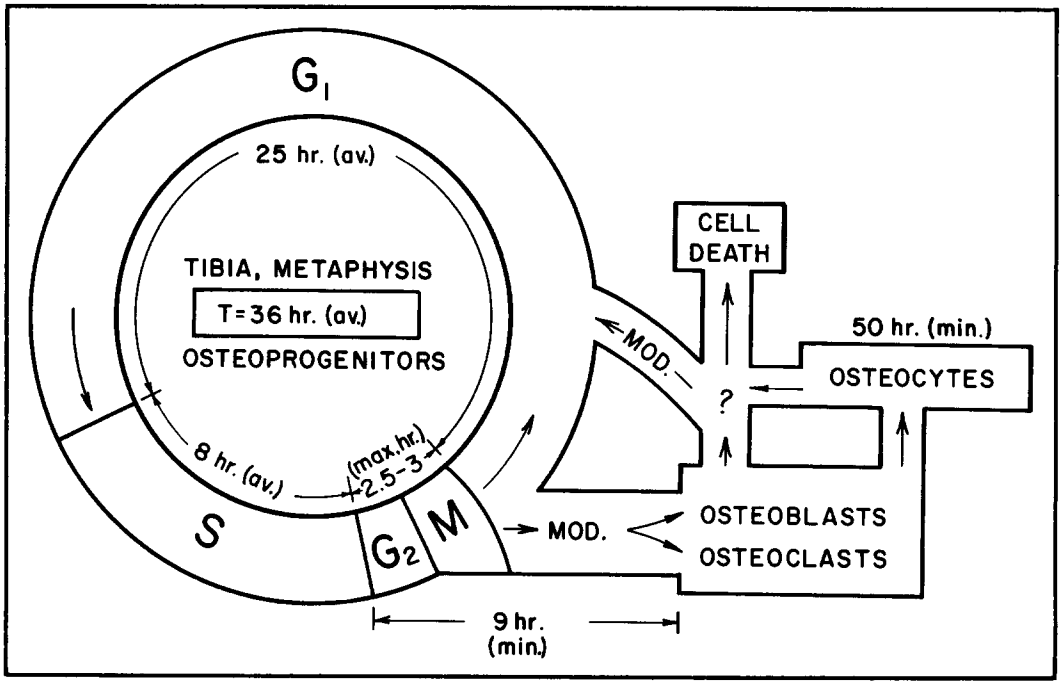

FIGURE 9

Diagram of cell proliferation and specialization in bones of young rats. Maximum (*max.*); minimum (*min.*); hours (*hr.*). Figures for the duration of progenitor cycle phases (left) are taken from the tibial metaphysis in which the average (*av.*) generation time (*T*) is about 36 hours. Following DNA synthesis (*S*), osteoprogenitor cells pass through G_2 and then mitosis (*M*). After division, the cells may (*a*) remain within the progenitor pool or (*b*) undergo modulation (*MOD.*) of cell type, specializing as osteoblasts or becoming incorporated in osteoclasts. Some osteoblasts will become enclosed in bone as osteocytes, there to remain until reached by resorption. Specialized bone cells may rejoin the progenitor population. Conceivably, some may be lost through cell death.

In summary, DNA is synthesized by osteoprogenitor cells in the latter part of interphase (Fig. 9). These cells then pass through a short G_2 period before undergoing mitosis. Following mitosis, the offspring have two major alternatives.

They may remain within the progenitor population or undergo modulation of cell type. In the latter case, they may specialize as osteoblasts or become incorporated in osteoclasts. Some osteoblasts will be trapped in bone as osteocytes, remaining until reached by resorption. Osteoblasts and osteoclasts probably return to the osteoprogenitor state as soon as the environmental stimulus which caused their formation is removed. Thus the conclusion appears inescapable that the different types of bone cells represent different functional states of the same basic cell (36).

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