CELL POPULATION KINETICS OF AN OSTEOGENIC TISSUE · I

MAUREEN OWEN, D.Phil.

From the Medical Research Council Bone-seeking Isotopes Unit, The Churchill Hospital, Oxford, England, and the Department of Biology, Brookhaven National Laboratory, Upton, New York

ABSTRACT

Cell proliferation on the actively growing periosteal surface of the femur of rabbits aged 2 weeks has been investigated using autoradiographic techniques. Injections of tritiated glycine and tritiated thymidine were given simultaneously and the animals sacrificed at intervals from 1 hour to 5 days after injection. The glycine labeled the position of the bone surface at the time of injection and the thymidine labeled the cells which were synthesising DNA. The rate of increase in the cell population was determined by counting the number of cells beyond the glycine label at different times after injection. The cell kinetics of the fibroblast-pre-osteoblast-osteoblast-osteocyte system has been studied. The fibroblasts are relatively unimportant from the point of view of increase in the cell population. The main site of cell proliferation is the layer of pre-osteoblasts on the periosteal surface. The rate of movement of cells from the pre-osteoblast to the osteoblast and osteocyte compartments has been measured. The incorporation of osteoblasts into the bone is not a random process, but it appears that the osteoblast must spend a certain time on the periosteal surface before becoming either an osteocyte or a relatively inactive osteoblast lining an haversian canal. It was estimated that, on an average, an osteoblast produces 2 or 3 times its own volume of matrix during its most active period on the periosteal surface.

INTRODUCTION

The uptake of tritiated thymidine into newly synthesised DNA by cells in preparation for division has been widely used in the study of cell proliferation in many tissues (1–3), and recently in bone (4–8). Cells which have incorporated the labeled thymidine into their nuclei can be located in sections by autoradiographic methods. Regions of proliferative activity can thus be recognised and the rate of movement of cells from one site to another or from one stage of differentiation to another can be investigated.

The present paper describes how the method has been applied to investigate the turnover of cells of the osteogenic connective tissue during the process of bone growth on the periosteal surface of the shaft of the femur of 2-week-old rabbits. Simultaneous injections of tritiated glycine and tritiated thymidine were given. The glycine served to mark the position of the surface of the bone matrix at the time of injection, the thymidine to label the cells which were synthesising DNA. Measurements were made of the total increase in cell population per day and also in the relative number of different cell types. The evidence showed that the main region of cell proliferation was the pre-osteoblastic layer on the periosteal surface, and the rate of cell division was estimated. The rate at which cells enter and leave the three compartments, pre-osteoblast, osteoblast, and osteocyte, and the approximate time of sojourn of

the cells in these compartments were determined. An estimate of the amount of bone matrix laid down per osteoblast was also made. In the paper that follows (paper II) a study is reported of the variation of the grain count spectra with time for the different cell types after injection of tritiated thymidine. Some interesting results on the uptake of thymidine without subsequent cell division in this system have been obtained.

MATERIALS AND EXPERIMENTAL TECHNIQUES

Dutch rabbits aged 2 weeks were used. The rabbits were suckling at the time of injection and they were returned to their mothers after injection. Injections were intraperitoneal and the specific activities of the solutions were as follows: tritiated thymidine, 1.9 c per mM; tritiated glycine 194 mc per mM. The amounts of isotope injected per gram of body weight were 0.33 μc of tritiated thymidine and 1.7 μc of tritiated glycine. The animals were killed at intervals up to 5 days after injection. Small pieces of the shaft of the femora were fixed in 10 per cent neutral formalin, decalcified in EDTA, and embedded in paraffin wax. Cross-sections, 7 microns thick, were cut. Autoradiographs of the sections were made using Kodak AR10 stripping film (9). The films were exposed for 5 weeks in a refrigerator at 4°C, developed for 7 minutes in D19B at 18°C, fixed for 15 minutes in Johnson's Fixsol (diluted 1:10), and washed in running water for 1 hour. Conditions were carefully standardised. The sections were stained through the photographic film with methyl green and pyronin.

METHOD FOR DETERMINING THE INCREASE IN CELL POPULATION

The periosteal surface was chosen as being a relatively simple *in vivo* system for the study of bone growth. In this region the cells are arranged in fairly well defined layers and the situation is not complicated by the presence of large amounts of marrow and cartilage as is the case in regions of growth in the metaphysis and endosteum. The growth over a time interval t on the periosteal surface of the shaft of the femur of a 2-week-old rabbit is represented diagrammatically in Fig. 1. In young rabbits the growing periosteal surface has a looped appearance characteristic of forming haversian systems. The surface is lined with osteoblasts and outside these there is a layer of pre-osteoblasts and beyond a layer of fibroblasts.

To study the cell kinetics of this system it is necessary to know the position of the periosteal surface at zero time. Injection of tritiated glycine was found to be an excellent label for this purpose. Glycine is naturally present in collagen in large amounts (10), and it has been shown previously (11) that injected glycine is rapidly metabolised by osteoblasts and incorporated into the bone matrix. In our system, tritiated glycine is present almost entirely in the matrix by about 4 hours after injection. At later intervals, it could be seen autoradiographically as a narrow band of grains between 10 and 20 microns wide which remains in place in the bone matrix (A in Fig. 1) outlining the position of the periosteal surface and to a lesser extent the neighbouring haversian canals at the time of injection (see also Figs. 3 and 8). The relative injected doses of thymidine and glycine were chosen so that the autoradiograph from glycine was just clearly distinguishable while the thymidine-labeled nuclei were easily visible. In counting the thymidine-labeled nuclei, those with 5 grains or more were counted as being labeled. This distinction between a labeled and an unlabeled cell is purely arbitrary. The glycine autoradiograph, at intervals greater than a few hours, *i.e.* after the main incorporation into the matrix, did not appreciably mask that from thymidine. In fact, the uptake of glycine was so rapid and discrete that the background of grains due to residual glycine in the region of bone formed after injection (between A and B, Fig. 1) was only two or three times the natural autoradiographic background. Since the latter was generally of the order of one grain per 100 square microns, this increase was not disturbing.

To determine the rate of increase in cell population, two injections, one of glycine and one of thymidine, were given simultaneously and the animals killed at intervals up to 5 days after injection. At any time interval t, all cells between the glycine band outlining the periosteal surface at time O (A, Fig. 1) and the first layer of fibroblasts must be either the same cells or daughter cells of the original layers of pre-osteoblasts and osteoblasts on the periosteal surface at time O. The layers of cells counted as being *on* the periosteal surface include those pre-osteoblasts and osteoblasts within the open loops and out to the first layer of fibroblasts (Fig. 1). The total cells at time t between the glycine band at A and the first layer of fibroblasts (Fig. 1) include the osteocytes embedded in the matrix, the cells in the haversian canals, and the layers of pre-osteoblasts and osteoblasts on the periosteal surface. The cells lining the surfaces of the canals have been counted as osteoblasts, whereas the cells within the canals but not on their surfaces have been counted as pre-osteoblasts.

Criteria for Distinguishing Cell Types

Part of the periosteal surface of a cross-section from the shaft of the femur of a 2-week-old rabbit is shown in Fig. 2. This animal had been given a single injection of tritiated thymidine and killed 1 hour later.

The osteocytes, *Oc*, because of their situation, each surrounded by bone matrix, were easily distinguished, and so also were the fibroblasts, *F*, which had typical, elongated, dense green nuclei with little or no cytoplasm. The osteoblasts, *Ob*, are very basophilic cells with a large amount of pink cytoplasm and pale green nuclei; the pre-osteoblasts, *POb*, cover all variations between the two extremes, from having dense green nuclei with little or no cytoplasm to having the appearance of typical osteoblasts. Distinction between osteoblasts and pre-osteoblasts was, therefore, not easy and the criterion used was simply as follows. Cells were designated osteoblasts if they were situated right up against the bone surface; otherwise they were called pre-osteoblasts. It is realised, therefore, that some of the cells counted as pre-osteoblasts may be fully differentiated osteoblasts and *vice-versa.*

Region of Bone Surface Studied

Growth on the periosteal surface of the bone depends on both the position along the length of the bone and the point on its circumference (12, 13). Since the experiment involves a comparison of results from one animal to another, care was taken to ensure that the same part of the bone surface was studied. The *adductor longus* has a tendon attachment to the femur about one-third of the way from its upper end. The attachment can be recognised by an indentation in the bone circumference which extends over a length of about 1 mm. Cross-sections were cut about midway through this region, and a portion of the bone surface about 300 microns distant on either side of this region was studied.

The number of pre-osteoblasts in the layers *on* the periosteal surface per unit length of arc of the circumference in this region, for different animals, is shown in Table I. The unit length (365 μ) of surface is chosen arbitrarily. It is the length which gave an average count of 100 osteoblasts for rabbit 12-19G. The range of variability among animals is about 10 per cent for the osteoblasts and 20 per cent for the pre-osteoblasts. The agreement was thus good enough to make the experiment feasible. Fig. 3 illustrates the method. It shows the glycine in the bone matrix 4 days after injection marking the position of the periosteal surface at the time of injection. This particular animal was injected with glycine only.

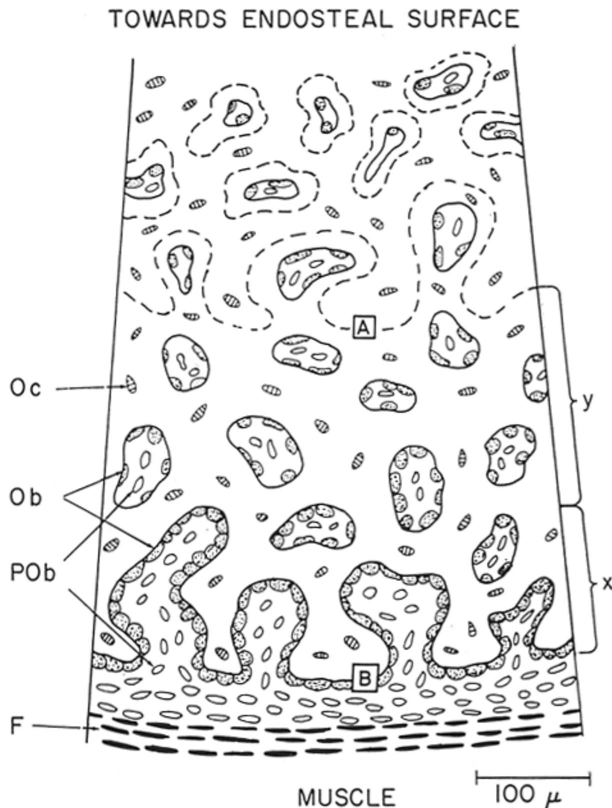

FIGURE 1 Diagram of bone growth on an arc of the periosteal circumference of the femur of a 2-week-old rabbit. *A* is the position of the periosteal surface at time *0* and *B* at time *t*. The uptake of glycine at time *0* is represented by the dotted lines. The arrow shows the direction of bone growth. *Oc*, osteocyte; *Ob*, osteoblast; *POb*, pre-osteoblast; *F*, fibroblast. Approximately to scale; $t = 4$ days.

RESULTS

Increase in Total Cell Population

The numbers of cells in the three layers (Fig. 1) on the periosteal surface were approximately in the ratio 1.5:1.0:0.6 for pre-osteoblasts, osteoblasts, and fibroblasts, respectively. In this investigation, detailed counts of the fibroblasts have not been made, since it was difficult in some cases to keep this layer intact. Enough measurements were made, however, to show that the number of fibroblasts per unit length of bone surface was constant during the 5 days and, as stated above, that their number was comparatively small, about 0.6 that of the osteoblasts and 0.4 that of the pre-osteoblasts. In addition, the percentage of fibroblasts which had incorporated thymidine at 1 hour was low, between 1 and 3 per cent. It is unlikely, therefore, that the fibroblasts play a large role in the increase in the cell population.

The increase in the total number of pre-osteoblasts, osteoblasts, and osteocytes, due to bone growth on an arc of the circumference (Fig. 1) in the periosteal region of the femur described above, has been measured by counting the number of cells at different times after injection. The increase in the total number of cells for rabbits injected at the age of 2 weeks and killed at intervals up to 5 days is shown in Fig. 4, in which length of the arc of the periosteal surface at 1 hour was 365 μ. At later times, the length of the arc on the surface is greater by about 3.5 per cent per day due to bone growth (see diagram Fig. 1). The total number of cells, pre-osteoblasts + osteoblasts + osteocytes, increased by about 20 per cent per day during the 5-day period. The increase in the number of cells in the different compartments, pre-osteoblasts, osteoblasts, and osteocytes, is shown in Fig. 5. It can be seen that the additional cells per day are divided among the three compartments in approximately the following way: 30 per cent of the extra cells are in the pre-osteoblasts, 41 per cent

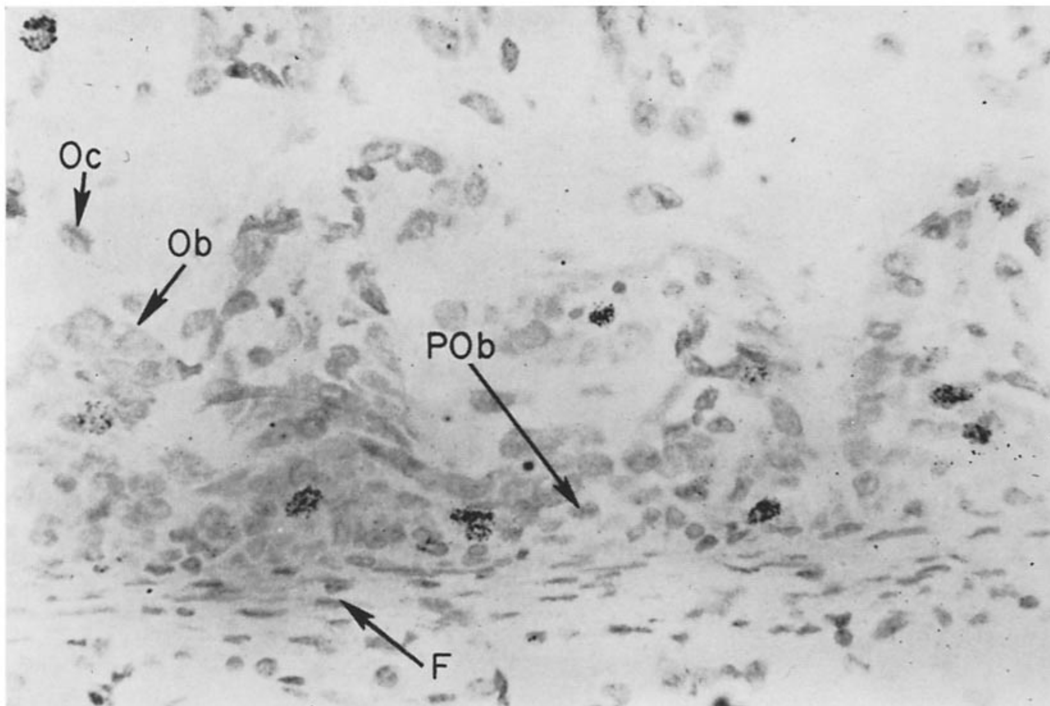

FIGURE 2 Part of the periosteal surface of a cross-section from the femur of a 2-week-old rabbit. The rabbit was given a single injection of tritiated thymidine and killed 1 hour later. Labeled cells are mostly pre-osteoblasts. *Oc*, osteocyte; *Ob*, osteoblast; *POb*, pre-osteoblast; *F*, fibroblast. Stain methyl green and pyronin. × 394.

TABLE I

No. of Cells per Unit Arbitrary Length (365 μ) of the Periosteal Surface, Cross-Section

Rabbit (Time killed)	Pre-osteo-blasts	Length of surface counted	Osteoblasts	Length of surface counted
12-19G (1 hour)	145	4.3 mm	100	4.3 mm
12-19E (2 days)	154	1.7 mm	104	2.8 mm
12-19D (3 days)	140	0.94 mm	112	0.94 mm
13-43C (4 days)	134	3.37 mm	98.4	3.37 mm
12-19F (5 days)	165	1.31 mm	99	2.62 mm

are in the osteoblasts, and 29 per cent are in the osteocytes.

In Table II the results for the pre-osteoblasts and osteoblasts are subdivided into cells *on* the periosteal surface and in the haversian canals. Although the number of cells *per unit length* of periosteal surface is approximately constant during the 5-day period (Table I) the number of cells on the periosteal surface, as shown in Table II, increases on average with time. This is due to the increase in the circumference of the bone with growth. The pre-osteoblasts and osteoblasts on the periosteal surface are thus an expanding population and, in addition, they are the main source of production of the cells which replace those which become included within the bone during the process of growth, *i.e.* the osteoblasts and pre-osteoblasts in the haversian canals and the osteocytes in the bone matrix. The number of labeled cells in each compartment and the approximate amount of bone growth are also shown in Table II.

The flow pattern for the cells in the present

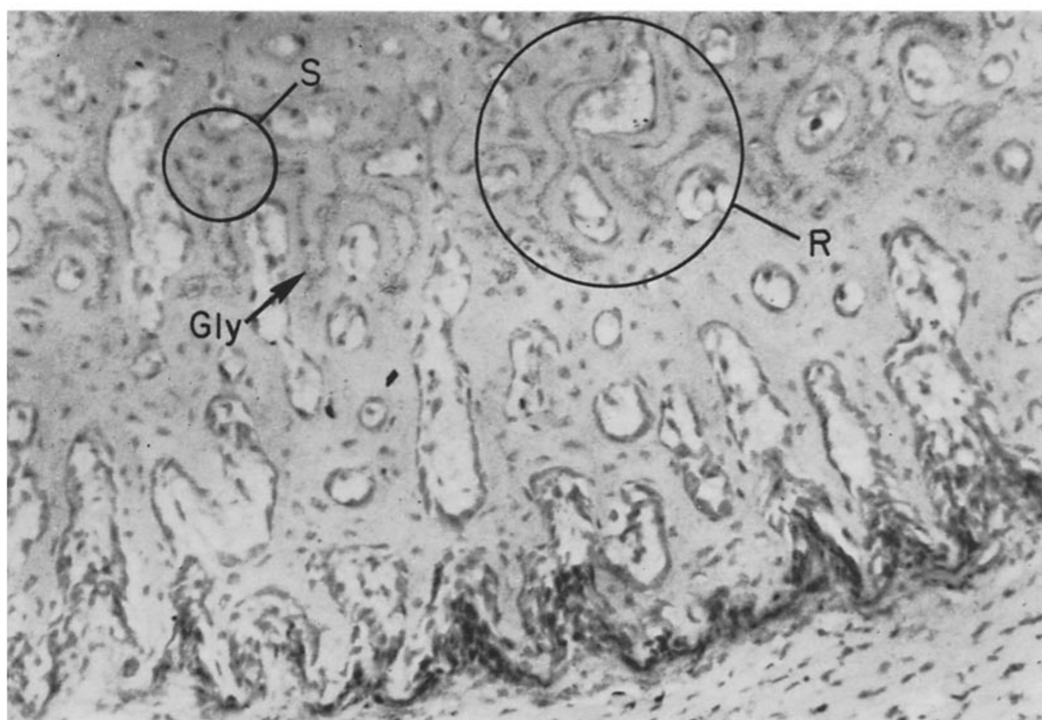

FIGURE 3 Part of the periosteal surface of a cross-section from the femur of a 2-week-old rabbit. The rabbit was given a single injection of tritiated glycine and killed 4 days later. The glycine (Gly) outlining the position of the periosteal surface and the neighbouring haversian canals at the time of injection is in the region indicated by the arrow. Detail of regions R and S is shown in Figs. 8 and 9. × 168.

system can be represented by the diagram in Fig. 6, in accordance with the scheme proposed by Quastler (14) for the description of cell population kinetics in a model system. The vertical lines represent intake of cells by birth and loss due to death in the various compartments. In the particular case being studied, the situation is greatly simplified by the fact that many of the possible routes for cell flow are ruled out, for various reasons. Those considered to be highly unlikely, such as the possibility of dedifferentiation over the time interval studied, are not included in Fig. 6. As will be seen, those represented by the dotted lines are also small or unlikely.

For the reasons given above, flow of cells to or from the fibroblast compartment must be relatively small and can be neglected. Loss of cells by death from any of the compartments is probably negligible, as will be seen from the evidence given later. Division of the osteoblasts and pre-osteoblasts in the haversian canals within the bone is also expected to be small since the canals are contracting in size. There may be a small amount of cell transfer via routes E and F (Fig. 6), but this is included in the measurement of the flow of cells along C and D and is not distinguishable from them. The solid lines (Fig. 6) represent the most likely transitions, and the values for them obtained from the present results are given below.

The number of cells added to the layers of pre-osteoblasts and osteoblasts on the periosteal surface by cell division or birth (routes X and Y, Fig. 6), must balance the number leaving the periosteal surface to become included within the bone (routes B, C, and D), except for a net increase of pre-osteoblasts and osteoblasts on the periosteal surface of about 3.5 per cent per day; this was determined by measuring the average increase in the bone circumference. The flow of cells per day from the surface to the compartments within the bone can be represented by the following equation.

$$K_X + K_Y - K_B - K_C - K_D = 0.035 (N_{PS} + N_{Obs}) \quad (1)$$

where $K_X \ldots$ etc. is the number of cells per day passing along route $X \ldots$ etc., and N_{PS} and N_{Obs} are the numbers of cells in the pre-osteoblast and

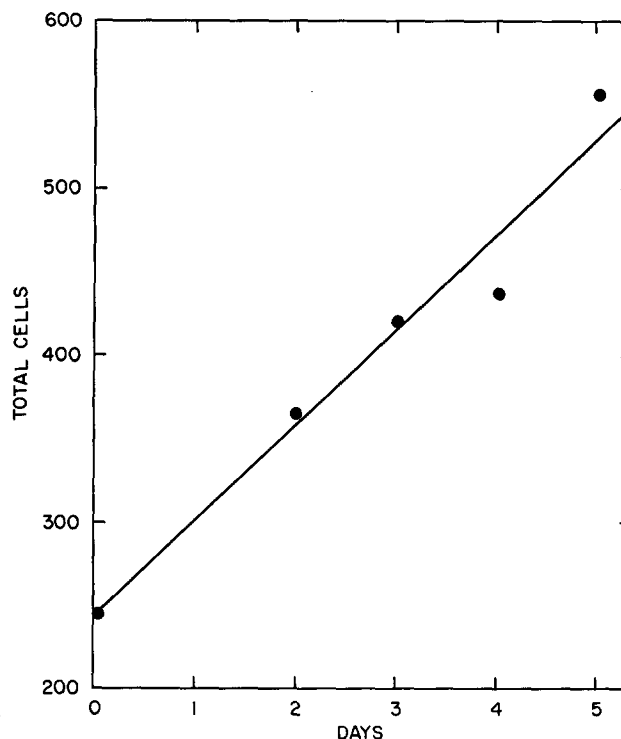

FIGURE 4 Increase with time in the total number of cells, pre-osteoblasts + osteoblasts + osteocytes, due to bone growth. The initial population unit at 1 hour was the pre-osteoblasts + osteoblasts on a length of the periosteal surface of 365 microns.

osteoblast compartments on the periosteal surface, respectively. K_B, K_C, and K_D are the numbers of cells per day added to each of the three compartments within the bone. The initial population unit is the pre-osteoblasts and osteoblasts on a 365-micron length of periosteal surface, and the increase in the number of cells in the various compartments at different times after injection, *i.e.* as the bone grows in width (Fig. 1), has been determined by direct counting (Table II). The average *i.e.* the division rate per day (or the birth-rate) of the pre-osteoblasts and osteoblasts, respectively, on the periosteal surface.

The osteoblasts lost from the periosteal surface must be replaced either (i) by division of the osteoblasts themselves, K_Y (Fig. 6), or (ii) by differentiation of the pre-osteoblasts into osteoblasts, K_A, or by a contribution from both (i) and (ii). The results on the distribution of thymidine-labeled cells described in the next paragraph show

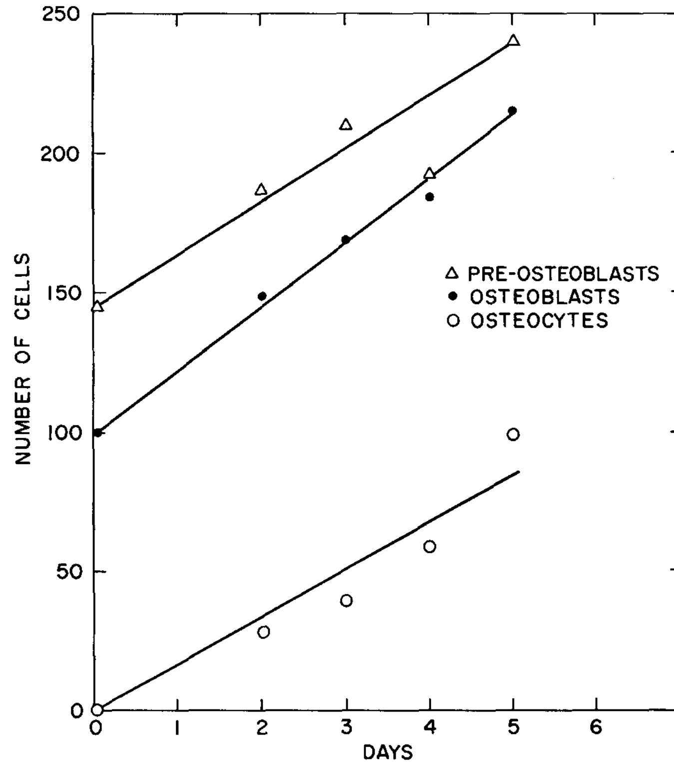

FIGURE 5 The results in Fig. 4 subdivided into pre-osteoblasts, osteoblasts, and osteocytes, illustrating the increase with time after injection in the number of cells in each category.

values over the 5-day period for K_B, K_C, and K_D and for N_{PS} and N_{ObS} have been determined. If the numerical values are put into equation (1), it can then be written

$$K_X + K_Y - 10.4 - 18.0 - 15.6 = 0.035\ (158 + 109)$$

therefore

$$K_X + K_Y = 53.3.$$

From the present measurements, it is not possible to determine the separate values of K_X and K_Y, that (ii) is certainly occurring, but it is not possible to say how much of (i) is taking place. The number of pre-osteoblasts differentiating into osteoblasts, *i.e.* the value of K_A, will depend on the relative values of K_X and K_Y. If, for the moment, it is assumed that there is zero division rate among the osteoblasts, *i.e.* $K_Y = O$, and that the total increase in cells is due to division of the pre-osteoblasts on the periosteal surface only, then $K_X = 53.3$ which means that the pre-osteoblasts on the periosteal surface must increase by about 33 per cent per day.

TABLE II

The Increase in Cell Population Due to Bone Growth on an Arc of the Circumference in the Periosteal Region

See Fig. 1, text and footnote

Rabbit (Time killed)	Pre-Osteoblasts				Osteoblasts				Osteocytes		Average width of band of bone growth μ	Total No. of cells counted in all categories
	No. of cells on periosteal surface	No. of labeled cells on periosteal surface	No. of cells in haversian canals	No. of labeled cells in haversian canals	No. of cells on periosteal surface	No. of labeled cells on periosteal surface	No. of cells in haversian canals	No. of labeled cells in haversian canals	No. of cells	No. of labeled cells		
12-19G (1 hour)	145	16.1			100	4.1						3040
12-19E (2 days)	164.8	17.3	22.4	1.04	111.3	10.4	37.5	2.1	28.6	0	174	3340
12-19D (3 days)	176.8	16.1	33.6	2.02	123.8	8.4	45.9	3.0	39.6	0.3	248	1378
13-43C (4 days)	152.8	7.7	39.5	3.1	112.2	11.6	72.8	5.1	59.4	2.7	267	3617
12-19F (5 days)	193.0	8.3	47.2	5.5	116.3	7.8	99.9	7.1	100	4.8	402	4280

The length of the arc of periosteal surface at 1 hour was 365 μ. Thereafter the length of the arc on the surface increased by about 3.5 per cent per day due to bone growth (see diagram, Fig. 1).

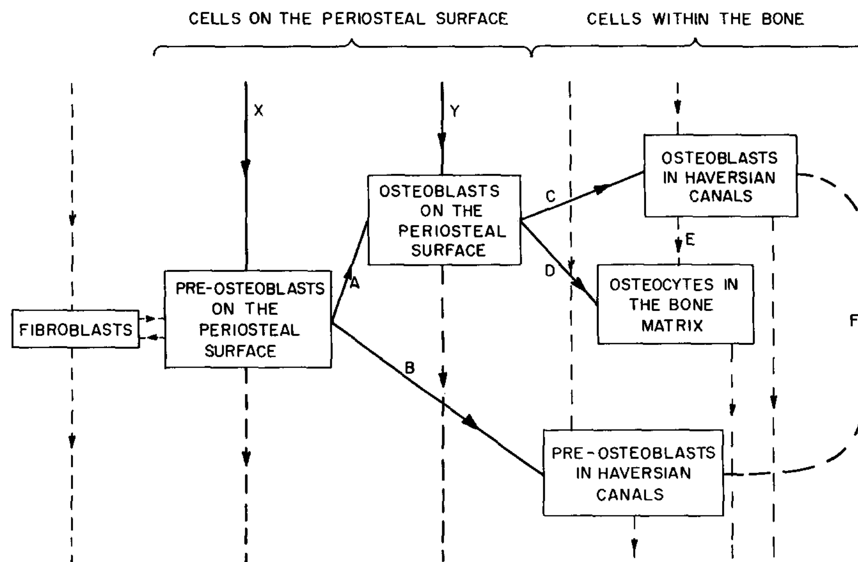

Figure 6 Diagram illustrating the transitions of cells due to bone growth on the periosteal surface (see text). The vertical lines represent intake of cells by birth and loss due to death in the various compartments. Cell flow along the dotted pathways is small or unlikely.

It is also evident ($K_C + K_D = 33.6$ cells per day) that about 31 per cent of the osteoblasts on the periosteal surface become included in the bone per day either as osteoblasts lining haversian canals or as osteocytes. On the assumption that there is no division of the osteoblasts, it can be concluded, therefore, that in the case of both the pre-osteoblasts and the osteoblasts the time spent by these cells on the periosteal surface is about 3 days.

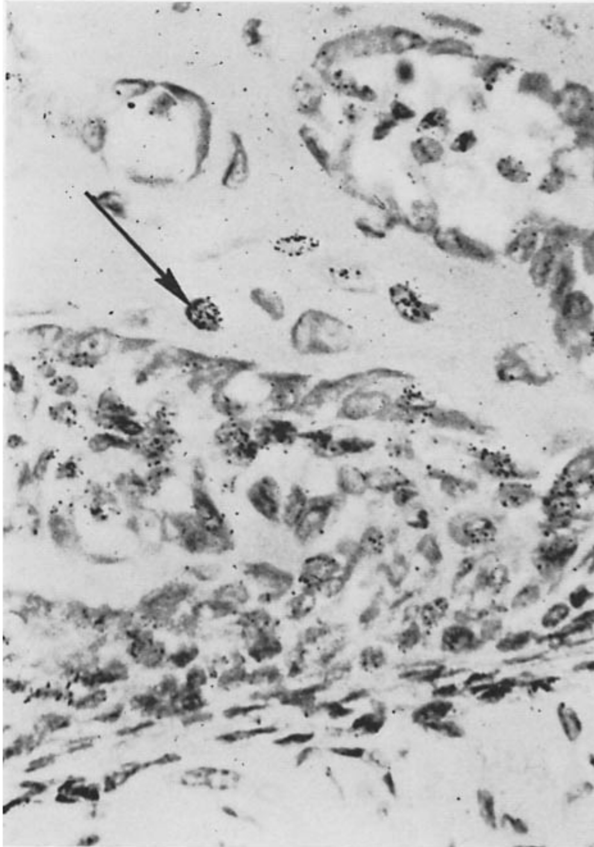

FIGURE 7 Part of the periosteal surface from the femur of a 2-week-old rabbit showing labeled osteocyte (arrow) 3 days after injection of tritiated thymidine. × 503.

Thymidine-Labeled Cells

The number and distribution of cells or their daughter cells, which are labeled with thymidine, and which were synthesising DNA at the time of injection, are shown in Table II. About 11 per cent of the pre-osteoblasts on the periosteal surface are labeled at a short time (1 hour) after injection. A small percentage of the osteoblasts, about 4 per cent, was also labeled at 1 hour; this could indicate a finite value for K_Y, *i.e.* some division of osteoblasts. There is evidence in the paper that follows (No. II), however, that the majority of these initially labeled osteoblasts have not divided (by 3 or 4 days, at any rate), and this is discussed in more detail there. The number of labeled cells in the osteoblast + osteocyte compartments increases fairly rapidly with time after injection, and at times greater than 2 days it is from $2\frac{1}{2}$ to 4 times the number of labeled osteoblasts at 1 hour after injection. Furthermore, the percentage of labeled cells in these compartments at times greater than 2 days varied from 1.4 to 2 times that at 1 hour. This increase in the percentage of labeled cells rules out the possibility that the labeled cells in the osteoblast + osteocyte compartments could be accounted for by division of labeled osteoblasts only. It is reasonable, therefore, to assume that the extra cells are supplied by feed-in from the pre-osteoblast compartment.

The number of labeled pre-osteoblasts on the periosteal surface stays approximately constant for the first 3 days. This would suggest that the numbers of cells entering and leaving the pre-osteoblast

compartment are about equal, provided the population is fairly constant over the period of time. This is the case since the increase per day, 3½ per cent, is small. At 4 and 5 days, there are signs of a fall in the number of pre-osteoblasts scored as labeled. This is also consistent with a turnover of pre-osteoblasts of about 33 per cent per day. By 4 and 5 days, many cells would be going through their second division and a high proportion of these would register less than 5 grains on an autoradiograph and, therefore, would not be counted.

Labeled osteocytes, examples of which are shown in Fig. 7, were not seen 2 days after injection; they had just appeared by 3 days and after that their number steadily increased. The actual experimental result was quite striking: at 3 or 4 days after injection the labeled osteocytes were seen in region *x* (Fig. 1) but never in region *y*, even though there were labeled osteoblasts on the surfaces of the haversian canals throughout *x* and *y*. This in itself is some indication that during this time most of the osteocytes are embedded directly from the periosteal surface and that the time spent by osteoblasts on the periosteal surface of the bone is about 3 days. It would appear that the osteoblasts in the haversian canals stay there for a much longer time, laying down bone at a relatively slow rate, and that they may remain there indefinitely or eventually be embedded as osteocytes.

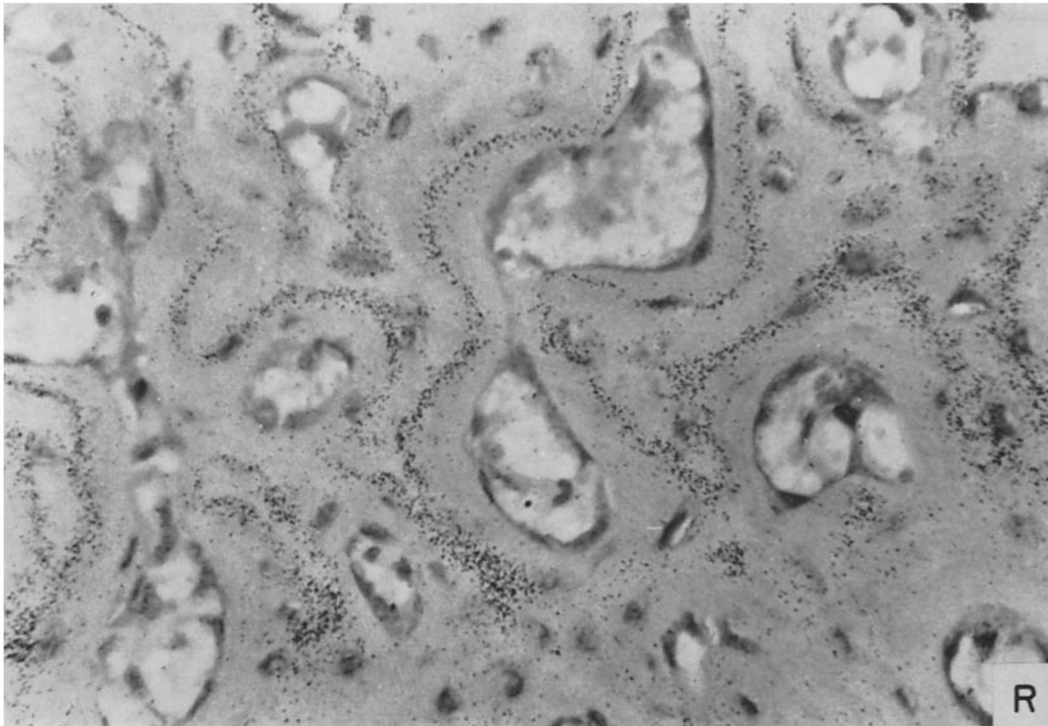

FIGURE 8 A high magnification of region *R*, Fig. 3. Shows the glycine band in the matrix outlining the periosteal surface and neighbouring haversian systems at the time of injection. × 440.

After 3 days, the percentage of labeled osteocytes which were incorporated each day was 12.1 and 5, respectively and the percentage of labeled osteoblasts on the periosteal surface at 3, 4, and 5 days was 6.8, 10.3, and 6.7, and for labeled osteoblasts in haversian canals, 6.5, 7.0, and 7.1. The fairly good agreement between these three sets of values gives one fair confidence in drawing the conclusion that in the period studied there is no loss of osteoblasts by cell death or otherwise and that in this system all osteoblasts proceed to become either osteoblasts in haversian canals or osteocytes in bone. In a similar way, there was no evidence for loss of pre-osteoblasts. No evidence of cell death was seen in the sections.

Bone Growth on the Periosteal Surface

Growth in this region of the periosteal surface of these young rabbits is extremely rapid (Fig. 3). Measurement of the approximate position of the front of the glycine label showed that a band of bone of average depth 78 microns was laid down per day (Table II). On the endosteal surface, resorption of the bone occurs at about the same rate. This rate of growth on the periosteal surface is approximately maintained until by about 10 days the glycine originally taken up there has reached the endosteal surface.

The glycine label is taken up throughout a depth of about 180 μ in a lace-like pattern around the haversian canals which are being formed on, or which are adjacent to, the bone surface (see Figs. 1 and 3). Growth continues on the surfaces of the haversian canals (see Fig. 8 which is detail from Fig. 3). However, as can be seen from the figures, the amount of matrix laid down around the haversian canals is small compared with the total amount added to the periosteal surface. The uptake of glycine in osteocytes which were already embedded in the bone matrix at the time of injection already has been reported (15), and this has also been observed in our material. An example of this is shown in Fig. 9, which is a high magnification of another part of Fig. 3. Radioactive glycine, mainly localised in the cytoplasm and possibly the adjacent matrix, can be seen in the osteocyte (arrow *1*). The bands of glycine uptake due to growth on the bone surfaces at the time of injection are seen at arrows *2* and *3*. Only the young osteocytes near the periosteal surface showed a positive autoradiograph at this dose level and exposure time. Nevertheless, this is evidence that osteocytes are not inert, and it is possible that osteocytes of all ages may be similarly capable of bone metabolism under certain circumstances.

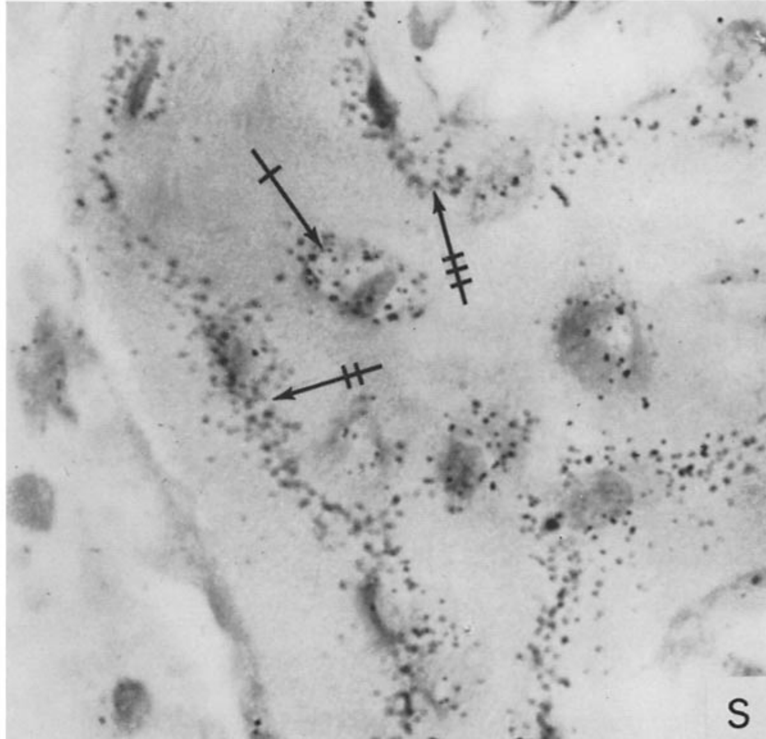

FIGURE 9 A high magnification of region *S*, Fig. 3. Illustrates glycine uptake in the cytoplasm of the osteocyte (arrow *1*). Bands of glycine taken up on the neighbouring bone surfaces are shown (arrows *2* and *3*). × 1007.

From the above measurements of the amount of bone laid down on the periosteal surface and the average time spent by an osteoblast on this surface,

it is possible to make a rough estimate of the amount of bone laid down per osteoblast. Approximately 100 osteoblasts line a 365-micron width of bone surface. The average time on the periosteal surface for an osteoblast is about 3 days and a band of bone about 78 microns wide is laid down per day. In the newly laid down band of bone the soft tissue spaces occupy about one-third of the area. If we take this into account and assume that the depth of an osteoblast is about 15 microns, then 858,000 cubic microns of bone are laid down by 100 osteoblasts in 3 days. An osteoblast has a volume of about 3,500 cubic microns, *i.e.* is approximately a cube of side 15 μ, hence each osteoblast manufactures about 2 to 3 times its own volume of matrix before leaving the periosteal surface to become either an osteocyte in the bone matrix or an osteoblast within a haversian canal. As an osteoblast lining a haversian canal, it will continue to lay down bone matrix, but, as shown above, the amount laid down will be small compared with that deposited during its time on the periosteal surface.

DISCUSSION

Although the role of the fibroblasts has not been fully worked out and requires further investigation, nevertheless there was sufficient information to show that the fibroblasts are a subpopulation of relatively small size with a low degree of labeling. They are likely, therefore, to play only a small role from the point of view of increase in cell population. Essentially the same conclusion was reached by Tonna and Cronkite (16) from their study of cell proliferation on the periosteal surface of young mice.

It was assumed that only the cells on the periosteal surface divide as opposed to those within the haversian canals. This assumption is probably justified, since the canals are contracting in size and also mitoses are much more rarely seen within the canals than among the cells outside the bone surface. Furthermore, more than 90 per cent of the pre-osteoblasts labeled at 1 hour after injection are on the periosteal surface (see paper II).

An initial high labeling of the pre-osteoblastic layer on the periosteal surface indicates that this is the main site of cell proliferation in this system. As defined in the present work, the pre-osteoblasts certainly consist of cells in different stages of differentiation (see paper II). They are also likely to include some endothelial cells which line the vessels on the periosteal surface and in the haversian canals. The pre-osteoblasts are probably equivalent to the mesenchymal cells or osteoprogenitor cells which have been described, by other authors (6, 8), as the proliferating section of the cell population in the metaphysis, and it would appear likely that they constitute the stem cells from which the other specialised cell types are derived.

An initial small labeling of osteoblasts by thymidine was found in this study and also by previous investigators (5–8). This result could be explained by a low rate of division of osteoblasts although it is commonly assumed that osteoblasts divide very little, if at all (17, 18). There is evidence in paper II, however, that the majority of these initially labeled osteoblasts do not divide, and it is assumed there that the rate of division of osteoblasts is zero. This means, then, as shown in the section on Results, that in order to account for the total increase in cell numbers it must be assumed that the pre-osteoblasts increase at the rate of about 33 per cent per day.

An interesting result was the fact that labeled osteocytes did not appear until at least 3 days after injection. Young (8) also found that labeled osteocytes were not seen before 2 days in the metaphysis of 6-day-old rats, and Kember (6) reported 5 days before the appearance of labeled osteocytes in the metaphysis of 6- to 8-week-old rats. These results would indicate that the embedding of osteoblasts in the bone matrix to form osteocytes is not a random process, but that the osteoblasts must spend a certain time on the bone surface before going on to become osteocytes. This is supported by the fact that in the present material the average time spent by the osteoblasts on the periosteal surface was measured and found to be about 3 days, which is the time interval that occurs before labeled osteocytes are seen.

It is quite likely that this time spent by an osteoblast on the periosteal surface is related to the amount of bone matrix laid down by the osteoblast. In a situation such as the present one where very active growth is taking place, the osteoblast lays down between 2 and 3 times its own volume of matrix in 3 days before settling down as an osteocyte or as a cell lining a haversian canal. It is interesting to speculate that cells in a less actively growing region where the matrix is being deposited at a slower rate may spend a proportionately longer time on the bone surface. Similarly,

the cell cycle time of the proliferating cells may be related to the rate of growth, though the evidence so far is meagre. For example, it was noted that Kember (6) found an average cell cycle time for the cells in the proliferative zone of the cartilage plate to be about 2 days and a growth to be about 100 μ per day, compared with 3 days and 78 μ per day for the proliferating pre-osteoblasts and growth of the periosteal surface in the present material. Differences in cell cycle times in the mesenchymal cells in the rat, depending on whether they were situated in the metaphyseal, endosteal, or periosteal regions, have been found recently (7, 8) and may well be related to different rates of production of matrix in these regions.

Finally, the present study has been made in a region where deposition of bone matrix or bone growth only is occurring, so that no information has been obtained on the possible interrelationships of these cells and other bone cells, for example, the osteoclast. It is clear that in the present system the region of pre-osteoblasts is the region of maximum proliferative activity and that they are the precursors of the osteoblasts which are in turn the precursors of the osteocytes, in agreement with classical histological observations. This system has many nice properties, among which is the fact that the final stage of the cell, the osteocyte, is preserved at least for a limited period. This has made it easier to follow the progress of the cells through the different functional stages and in theory to account for the fate of all cells. In practice, this turned out to be possible and it was found that the rate of production of cells in the proliferative compartment was balanced by the increase in the number of cells in this compartment and loss of cells through the process of differentiation. The conclusion was reached that, in this actively growing system over the time interval studied, there was little or no loss of cells by cell death and that all osteoblasts proceed to become either osteocytes in the bone matrix or osteoblasts within the haversian canals.

The author is at the Biology Department, Brookhaven National Laboratory, on leave of absence from the Medical Research Council, The Churchill Hospital, Oxford, England, from November 1962–1963. A portion of this research was performed at Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.

The author is particularly indebted to Dr. Henry Quastler for many valuable suggestions and discussions on the contents of both papers I and II. She is also very grateful to Dr. Stephen Pelc and Dr. Janet Vaughan for their helpful advice throughout the course of this work. Finally, she would like to thank Mrs. Jeanne Hampton and Miss Veronica Merrick for excellent technical assistance.

Received for publication, February 21, 1963.

REFERENCES

1. Hughes, W. L., Bond, V. P., Brecher, G., Cronkite, E. P., Painter, R. B., Quastler, H., and Sherman, F. G., Cellular proliferation in the mouse as revealed by autoradiography with tritiated thymidine, *Proc. Nat. Acad. Sc.*, 1958, **44,** 476.
2. Quastler, H., and Sherman, F. G., Cell population kinetics in the intestinal epithelium of the mouse, *Exp. Cell Research*, 1959, **17,** 420.
3. Leblond, C. P., Messier, B., and Kopriwa, B., Thymidine-^{3}H as a tool for the investigation of the renewal of cell populations, *Lab. Invest.*, 1959, **8,** 296.
4. Tonna, E. A., Osteoclasts and the aging skeleton: A cytological, cytochemical and autoradiographic study, *Anat. Rec.*, 1960, **137,** 251.
5. Tonna, E. A., The cellular complement of the skeletal system studied autoradiographically with tritiated thymidine ($H^{3}TDR$) during growth and aging, *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 813.
6. Kember, N. F., Cell division in endochondral ossification, *J. Bone and Joint Surg.*, 1960, **42B,** 824.
7. Young, R. W., Regional differences in cell generation time in growing rat tibiae, *Exp. Cell Research*, 1962, **26,** 562.
8. Young, R. W., Cell proliferation and specialization during endochondral osteogenesis in young rats, *J. Cell Biol.*, 1962, **14,** 357.
9. Pelc, S. R., The stripping film technique of autoradiography, *Internat. J. Appl. Rad. and Isotopes*, 1956, **1,** 172.
10. Eastoe, J. E., The organic matrix of bone, *in* The Biochemistry and Physiology of Bone, (G. H. Bourne, editor), New York, Academic Press, Inc., 1956, 81.
11. Carneiro, J., and Leblond, C. P., Role of osteoblasts and odontoblasts in secreting the collagen of bone and dentin, as shown by radioautography in mice given tritium-labelled glycine, *Exp. Cell Research*, 1959, **18,** 291.

12. OWEN, M., JOWSEY, J., and VAUGHAN, J., Investigation of the growth and structure of the rabbit tibia using autoradiographic and microradiographic techniques, *J. Bone and Joint Surg.*, 1955, **37B,** 324.
13. PONLOT, R., *in* Le Radiocalcium dans l'étude des os, Editions Arsica, Bruxcelles, 1959.
14. QUASTLER, H., Cell population kinetics, *Ann. New York Acad. Sc.*, 1960, **90,** 580.
15. YOUNG, R. W., Autoradiographic studies on postnatal growth of the skull in young rats injected with tritiated glycine, *Anat. Rec.*, 1962, **143,** 1.
16. TONNA, E. A., and CRONKITE, E. P., Autoradiographic studies of cell proliferation in periosteum of intact and fractured femora of mice utilizing DNA labelling with ^{3}H-thymidine, *Proc. Soc. Exp. Biol. and Med.*, 1961, **107,** 719.
17. PRICHARD, J. J., A cytological and histochemical study of bone and cartilage formation in the rat, *J. Anat.*, 1952, **86,** 259.
18. PRITCHARD, J. J., The osteoblast, *in* The Biochemistry and Physiology of Bone, (G. H. Bourne, editor), New York, Academic Press, Inc., 1956, 179.