CELL POPULATION KINETICS OF AN OSTEOGENIC TISSUE · II

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ABSTRACT

A study of the cell kinetics on the actively growing periosteal surface of the femur of rabbits aged 2 weeks has been continued. A single injection of tritiated thymidine was given and the rabbits killed from 1 hour to 4 days after injection. The grain count spectra of the different cell types, pre-osteoblast, osteoblast, and osteocyte, have been compared at different times after injection. The results showed evidence for the uptake of thymidine in nuclei which is not associated with cell division. A small percentage of osteoblasts was initially labeled at 1 hour and there was evidence that the majority of these had not divided by 3 or 4 days after injection. Some thymidine-labeled cells had also become osteocytes without division. Furthermore, it appeared that a considerable fraction of the initially labeled pre-osteoblasts did not divide. The S period for the pre-osteoblasts and osteoblasts was measured using a double-labeled thymidine technique.

INTRODUCTION

In the previous paper, I, cell proliferation on the growing periosteal surface of the femur of 2-week-old rabbits was studied using tritiated thymidine and tritiated glycine. The increase in the total cell population was measured directly by counting the number of cells produced within a certain time interval up to a maximum of 5 days. The main region of proliferative activity was the pre-osteoblasts on the periosteal surface. The rate of formation of osteoblasts from pre-osteoblasts and osteocytes from osteoblasts was also determined.

An unexpected result, noted in paper I, was the initial uptake of tritiated thymidine in osteoblasts, since it is commonly thought that these cells do not divide. In the region studied, about 4 per cent of them were labeled 1 hour after injection. Other authors have similarly found initial labeling of osteoblasts in the bones of rats (1–3) though at a lower rate, about 2 per cent. In this paper a study has been made, on material similar to that used in the investigations in paper I, of the variation in the grain count spectra with time for the three different cell types, pre-osteoblast, osteoblast, and osteocyte, following uptake of tritiated thymidine, in an effort to obtain information as to whether all cells which have taken up thymidine proceed through cell division. The results showed that the majority of the initially labeled osteoblasts have not divided by 3 or 4 days and, furthermore, that some cells which have taken up thymidine proceed to become osteocytes without division. In addition, it was found that a considerable fraction of the labeled cells designated as pre-osteoblasts had not divided. The period of DNA synthesis has been measured.

MATERIALS AND METHODS

Materials and methods, which are not described here, are the same as those in paper I. The slides for determination of the grain count spectra were exposed for 18 days. This gave a maximum grain count of about 60 grains per cell for 2-week-old rabbits (about

150 gm weight) injected with 0.33 μc/gram body weight of tritiated thymidine. Cells with five grains or more were scored.

Measurement of the S Period

An estimate of the S period, or period of DNA synthesis, for pre-osteoblasts and osteoblasts has been made by using the double-labeled thymidine technique described by Wimber and Quastler (4). Two rabbits were each given a single injection of ^{3}H-thymidine followed 2½ hours later by an injection of ^{14}C-thymidine and then killed 1 hour later. Because of the different ranges of beta particles from ^{3}H and ^{14}C, cells labeled with ^{3}H only can be distinguished autoradiographically from the other two types of labeled cells, *i.e.* those labeled with ^{14}C only or with (^{14}C + ^{3}H). Out of the total number of labeled cells, the proportion labeled with tritium only can be measured and the S period calculated from the relationship between this and the time interval between injection of the two labels. The theory of the method is given in detail in the original reference.

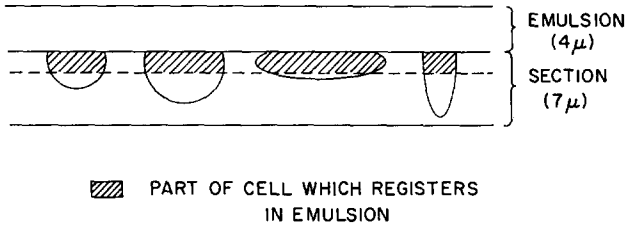

FIGURE 1 Diagram illustrating the differences, due to size, shape, and orientation of the nucleus, in the maximum volume of the nucleus which registers in the emulsion.

RESULTS

Distribution of Grain Counts over Labeled Nuclei in Sections

Grain counts over tritium-labeled nuclei, even when they are whole nuclei such as in squashes or smears, have a wide distribution (5). The most important factors contributing to this are (i) the very short range and consequently the high self-absorption of the β particles (maximum range in tissue about 8 microns, median range about 1 micron), which means that only the first 1 or 2 microns next to the emulsion are effective in producing an autoradiograph, (ii) the random nature of the β particle emission and (iii) the possibility that the uptake of thymidine may vary throughout the synthesis period of the cell cycle. In sections, the situation is further complicated since the effectiveness of the slice of nucleus which will register in the autoradiograph will depend on the size, shape, and orientation of the nucleus in the section. Fig. 1 illustrates diagrammatically the sort of variation which could occur in the maximum volume of the part of the nucleus in a section which is effective in producing an autoradiograph.

The region of growth on the periosteal surface studied was similar to that described in paper I. Both cross-sections and longitudinal sections, cut in the direction of the long axis of the bone, were examined. In longitudinal sections the cells and nuclei in the outer layer near the fibroblasts are very elongated in the direction of the bone axis, whereas farther in, within the bone and in the haversian canals, the nuclei are slightly larger and more rounded in shape. Some interesting differences were obtained when the grain counts per cell for the longitudinal sections were recorded according to their position with reference to the periosteal surface. Part of the periosteal surface in a longitudinal section is shown diagrammatically in Fig. 2. Region *O*, Fig. 2, was arbitrarily chosen to include approximately the cells between the fibroblastic layer and the first bone surface and the cells in the bottom halves of the loops. Region *I* includes the cells in the tops of the loops and the haversian canals. The results up to 4 days after injection for the mean grains per cell and the relative distribution of the cells in these two regions are shown in Table I.

At 1 hour after injection, when practically no cell division will have occurred, several interesting observations were made. In the first place, there appeared to be a definite regional difference in the number of grains per cell for both pre-osteoblasts and osteoblasts, the cells in the outer region, *O*, near the fibroblasts having lower grain counts than those in the inner region, *I* (Table I). This is probably due to the different orientation of the cells in the respective regions. There was no significant difference in mean grains per cell between pre-osteoblasts and osteoblasts in the same region at 1 hour after injection.

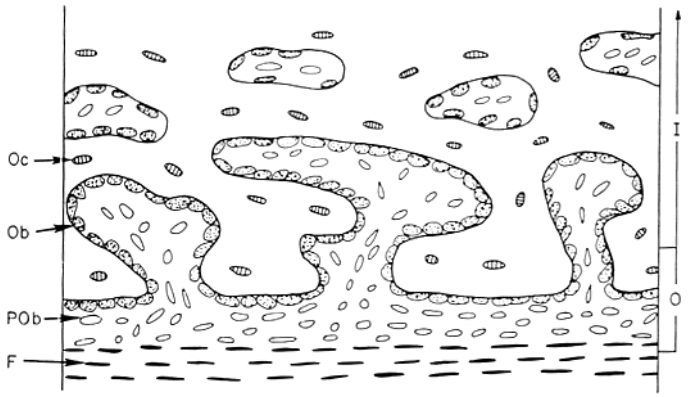

FIGURE 2 Diagram of part of the periosteal surface of a longitudinal section of the femur of a 2-week-old rabbit showing positions of regions *I* and *O* (see text). *Oc*, osteocyte; *Ob*, osteoblast; *POb*, pre-osteoblast; *F*, fibroblast.

TABLE I

Distribution of Labeled Cells and Mean Grains per Labeled Cell in Different Regions of the Periosteal Surface (See Fig. 2) (Longitudinal Sections)

	Pre-Osteoblasts				Osteoblasts			
	Region *O*		Region *I*		Region *O*		Region *I*	
Rabbit (Time killed)	No. of cells	Mean grains per cell	No. of cells	Mean grains per cell	No. of cells	Mean grains per cell	No. of cells	Mean grains per cell
12-19G (1 hour)	214	18.0	37	23.7	93	19.6	112	24.2
13-00A (1 hour)	172	19.2	33	20.5	117	19.7	91	24.0
13-00B (1 day)	217	14.9	93	16.2	57	14.6	63	19.6
13-00C (2 days)	134	11.2	51	14.1	92	13.8	36	17.9
13-00D (3 days)	145	9.9	96	14.0	72	13.6	140	18.4
13-00E (4 days)	104	9.4	162	12.0	63	11.1	137	17.2

The relative distribution of the cells in the two regions, however, was different for the two cell types. About 85 per cent of the labeled pre-osteoblasts are in region *O* at 1 hour after injection, whereas the osteoblasts are about equally distributed between the two regions. In fact, it was estimated that more than 90 per cent of the labeled pre-osteoblasts at 1 hour were on the periosteal surface, *i.e.* within the first loops out to the fibroblastic layer; this indicates that there is relatively little division of pre-osteoblasts within haversian canals and that the region of active proliferation of the pre-osteoblasts is the layer on the periosteal surface (see paper I).

In Table II the mean grains per nucleus on cross-sections is compared with that on longi-

tudinal sections. It can be seen that the mean grains per pre-osteoblast was less in cross-sections that in longitudinal sections though at the time interval of 1 hour only. This is probably due to differences in the portion of nucleus seen by the emulsion in longitudinal and cross-sections in the outer region *O* (Fig. 2). For osteoblasts the mean grains per cell was not significantly different in cross-sections and longitudinal sections. The mean grains per nucleus for pre-osteoblasts was lower than for osteoblasts in both longitudinal and cross-sections (Table II), and this is probably due to the different distributions of the two cell types in the various regions. As shown above, in longitudinal sections there was no difference when the cells were counted according to the region in which they were situated.

Grain counts in animals injected with the same amount of thymidine per gram body weight and killed at the same time interval are highly reproducible. In five rabbits killed at 1 hour after injection the mean grain count for 200 cells had a range of 25 per cent and the standard deviation among the five animals was ±9 per cent. These animals were not litter mates and were injected with different batches of thymidine and the autoradiographs made at different times; the conditions for reproducibilty were, therefore, at their worst. On the other hand, for two litter mates killed at 1 day, 12–19A and C, Table II, the agreement to within 1 per cent for the mean grains per cell was perhaps fortuitously good. In an experiment where the grain counts at different time intervals were to be compared from one animal to another, litter mates were used and the animals were injected and the autoradiographs prepared under the same conditions.

TABLE II

Variation with Time after Injection of the Mean Grains per Labeled Cell for Different Cell Types

	Cross-Sections						Longitudal Sections					
	Pre-Osteoblasts		Osteoblasts		Osteocytes		Pre-Osteoblasts		Osteoblasts		Osteocytes	
Rabbit (Time killed)	No. of cells counted	Mean grains/ cell	No. of cells counted	Mean grains/ cell	No. of cells counted	Mean grains/ cell	No. of cells counted	Mean grains/ cell	No. of cells counted	Mean grains/ cell	No. of cells counted	Mean grains/ cell
12-44A (1 hour)	137	17.8	126	22.6			—	—	—	—		
12-19G (1 hour)	221	14.6	202	19.8			251	18.9	205	22.1		
13-00A (1 hour)	241	16.7	252	24.4			205	19.4	208	21.8		
12-19A (1 day)	207	16.1	64	19.8			—	—	—	—		
12-19C (1 day)	221	16.0	62	18.6			—	—	—	—		
13-00B (1 day)	182	15.3	89	20.7			310	15.3	120	17.3		
13-00C (2 days)	137	14.0	132	18.0			185	13.4	128	16.8		
13-00D (3 days)	84	11.1	112	16.7	230	18.0	241	11.5	212	16.7	139	15.8
13-00E (4 days)	165	11.2	154	16.7	175	16.6	266	11.0	200	15.3	107	14.2

Mean Grains per Cell

Description of the results will be restricted to those from longitudinal sections since these are probably less dependent on the orientation of the cells in the tissue. Although the grain counts on cross-sections differed slightly from those on longitudinal sections as described in the previous section, they are compatible, however, with the conclusions drawn below. The results discussed are mainly for the series 13-00A to E, which were litter mates injected at the same time and killed from 1 hour up to 4 days after injection. The data illustrated by the results from this litter have been confirmed in a total of three separate experiments involving three different litters.

Variation in grain count spectra or fall-off in the mean grains per cell with time after injection is due to the halving of the number of grains per cell following cell division. The mean grains per cell for both the pre-osteoblasts and the osteoblasts decreases with time (Table II), though less rapidly than might be expected. In an actively proliferating tissue which has taken up thymidine following a single injection, it is reasonable to assume that all the labeled cells will have divided at least once, by 1 day, and very likely sooner, after injection. From the grain count distribution spectrum at 1 hour, the spectrum to be expected after all cells have divided once can be calculated, and consequently also the mean grain count. For the 1300 series the mean grain count, after all pre-osteoblasts and osteoblasts had gone through one division, was calculated to be 10.6 and 11.5 per cell, respectively. This is slightly higher than half the actual mean grain number, *i.e.* 9.7 and 10.9, respectively, due to the exclusion of a small precentage of weakly labeled cells (of the order of 10 per cent) which will have less than 5 grains after division and would, therefore, not be counted. The experimental values found for the mean grains per cell at 1 day after injection were 15.3 and 17.3 for the pre-osteoblasts and osteoblasts, respectively (Table II), which are significantly higher than those predicted if all the cells had divided once, and it was noted that the mean grains per cell for the osteoblasts remained high at 2, 3, and 4 days after injection. The mean grains per cell for the osteocytes (Table II) is also significantly higher than the estimated half grain number to be expected after cell division.

This analysis of the variation in mean grain counts is for the combined cells in regions *I* and *O*. Within either region, however, the mean grain counts have not fallen to half the value at 1 hour, except in region *O* for the pre-osteoblasts at 2 days and later, and for the osteoblasts at 4 days. The apparent difference in mean grain counts according to whether the cells are in regions *I* or *O* and the different distribution of the cells with time are not sufficient to affect the conclusions drawn below.

Grain Count Distributions

The grain count histograms corroborate the results for the mean grains per cell and yield further information. Typical grain count spectra at different times after injection for pre-osteoblasts and osteoblasts are shown in Fig. 3. It can be seen that, for both the pre-osteoblasts and the osteoblasts, there is an increase with time in the relative number of cells with low grain numbers but also the persistence of cells with grain counts of the same order of magnitude as the maximum grains per cell seen at 1 hour. This was true at all time intervals in the case of the osteoblasts (see also Fig. 4), but was also significant at 1 day in the case of the pre-osteoblasts. This indicates, therefore, that although there is a considerable amount of cell division particularly among the pre-osteoblasts, some pre-osteoblasts and osteoblasts which have taken up thymidine do not divide.

In the course of these experiments many measurements were made of the number of pre-osteoblasts and osteoblasts per unit length of bone surface at different times after injection compared with 1 hour, and these are given in Table III. They are in agreement with and include the data from paper I. The evidence for this feature of nondividing cells is seen most clearly from a comparison of the grain count histograms for the osteoblasts at 1 hour with those at later times, for the cells on the same length of bone surface. If we ignore the relatively small increase in bone circumference (about 3.5 per cent per day, paper I), then, for a certain length of bone surface, the labeled osteoblasts and osteocytes at later time intervals will have been derived from the initially labeled osteoblasts and pre-osteoblasts on the same length of bone surface at 1 hour. A typical example is shown, in Fig. 4, of the comparison of the grain count spectrum for osteoblasts at 3 days, rabbit 13-00L, with that of its litter mate, 13-00J, killed at 1 hour. There are about three times as many

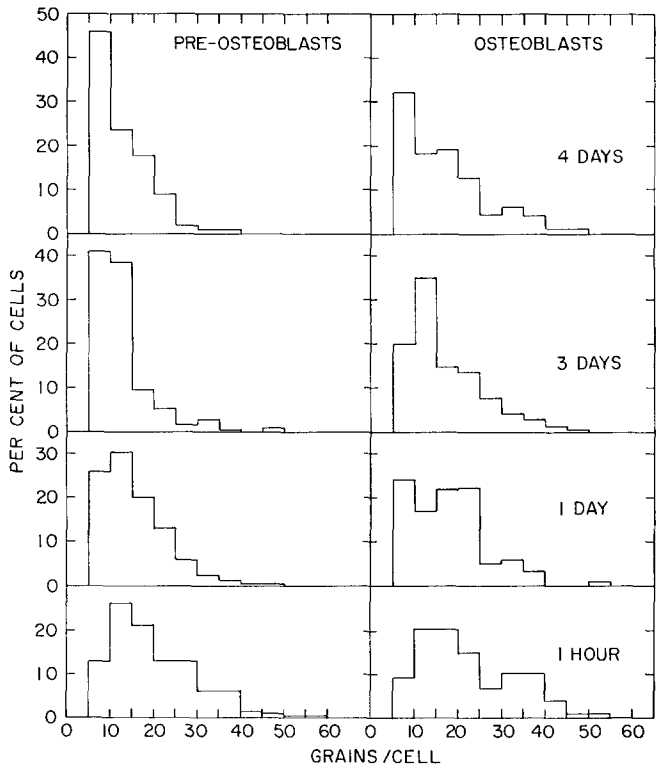

FIGURE 3 Grain count histograms for pre-osteoblasts and osteoblasts at different times after a single injection of tritiated thymidine.

TABLE III

Ratio of Labeled Cells at Time t to 1 Hour for the Same Unit Length of Periosteal Surface

Rabbit	Time killed	(1) Pre-Osteoblasts: Labeled cells at time t / Labeled cells at 1 hour	(2) Osteoblasts + Osteocytes: Labeled cells at time t / Labeled cells at 1 hour	(3) Pre-Osteoblasts + Osteoblasts + Osteocytes: Labeled cells at time t / Labeled cells at 1 hour	Type of section*
13-00B	1 day	1.12	1.20	1.14	L
10-15D	1 day	1.30	1.80	1.43	C
13-00C	2 days	0.84	2.85	1.24	L
12-19E	2 days	1.07	2.88	1.43	C
12-19D	3 days	1.02	2.60	1.31	C
13-00D	3 days	1.00	4.76	1.76	L
13-00L	3 days	1.09	3.52	1.58	C
13-43C	4 days	0.60	4.24	1.33	C
13-00E	4 days	0.60	2.80	0.94	L
12-19F	5 days	0.76	4.30	1.48	C

* L = longitudinal; C = cross-section.

labeled osteoblasts for the same length of surface at 3 days as at 1 hour.

The points which emerge from the comparison for the osteoblasts in Fig. 4 are as follows. The spectrum at 3 days shows that the main increase in the number of osteoblasts is due to the addition of cells at low grain numbers. This would be consistent with incoming pre-osteoblasts which have first divided and then gone on to become osteoblasts. The other feature of the spectrum is the tail of cells at 3 days with high grain counts. The number of labeled osteocytes for the same unit length of surface is of course much smaller, between 5 and 10 per cent of the number of labeled osteoblasts. However, for the purpose of comparison, in Fig. 4 the grain count spectrum for the osteocytes for two animals at 3 days has been plotted for the same number of cells as the osteoblasts. As can be seen, the spectrum for the osteocytes has essentially the same shape as for the osteoblasts and has a similar tail at high grain counts. In the case of both the osteoblasts and osteocytes, this tail of cells has grain counts too high for cells which have divided.

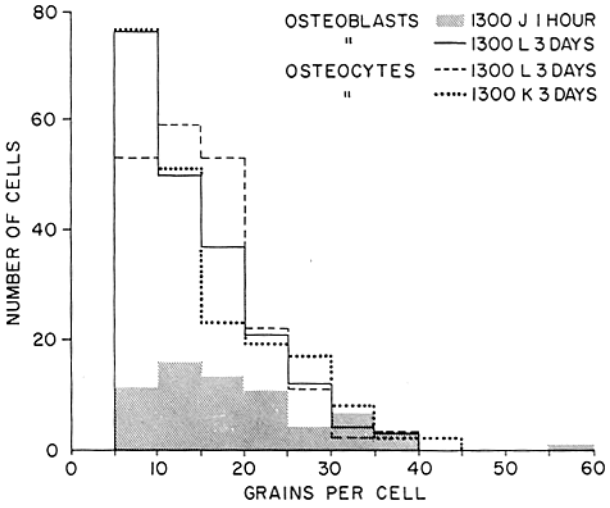

FIGURE 4 Grain count histograms for osteoblasts at 1 hour and 3 days after a single injection of tritiated thymidine for the same arbitrary length of bone surface. For comparison, histograms of osteocytes at 3 days, scaled to the same number of cells as the osteoblasts, are also shown.

The fraction of osteoblasts which this tail of high grain counts comprises is consistent with the assumption that the proportion of cells which do not divide is of the same order of magnitude as the number of initially labeled osteoblasts. For example, referring again to Fig. 3, at 1 hour after injection about 26 per cent of the osteoblasts have more than 30 grains per cell, at 3 and 4 days about 10 per cent. On the other hand, at 3 and 4 days there are about 3 times as many labeled osteoblasts. This figure of 10 per cent is equivalent, therefore, to about 30 per cent of the initially labeled osteoblasts, which agrees well with the value of 26. In the case of the pre-osteoblasts, the size of the fraction of the labeled pre-osteoblasts which do not divide within the 1st day after injection is considered in the Discussion section.

It is also of interest to look at the regional distribution of the pre-osteoblasts and osteoblasts at the later time intervals compared with that at 1 hour. When the grain count spectra were plotted separately for regions *I* and *O* (Fig. 2), it was found that for both cell types most of the cells with high grain numbers at the later time intervals were in region *I*. An example illustrating this for the osteoblasts is shown in Fig. 5 where the grain count spectrum from 13-00A, 1 hour after injection, is compared with that from 13-00E, 4 days after injection. The majority of the cells with high grain numbers at 4 days are found in region *I*, *i.e.* mostly in the haversian systems within the bone.

S Period Measurement

Measurement of the time of synthesis of DNA, or S period, by double labeling of the cells with ^{3}H- and ^{14}C-labeled thymidine, depends on one's being able to distinguish ^{3}H-labeled cells from ^{14}C- or (^{14}C + ^{3}H)-labeled cells. Examples of the two types of labeled cells are shown in Fig. 6, where *a* shows an osteocyte labeled with tritium only, and *b* shows a pre-osteoblast labeled probably with (^{14}C + ^{3}H). Because of the wide scatter of grains

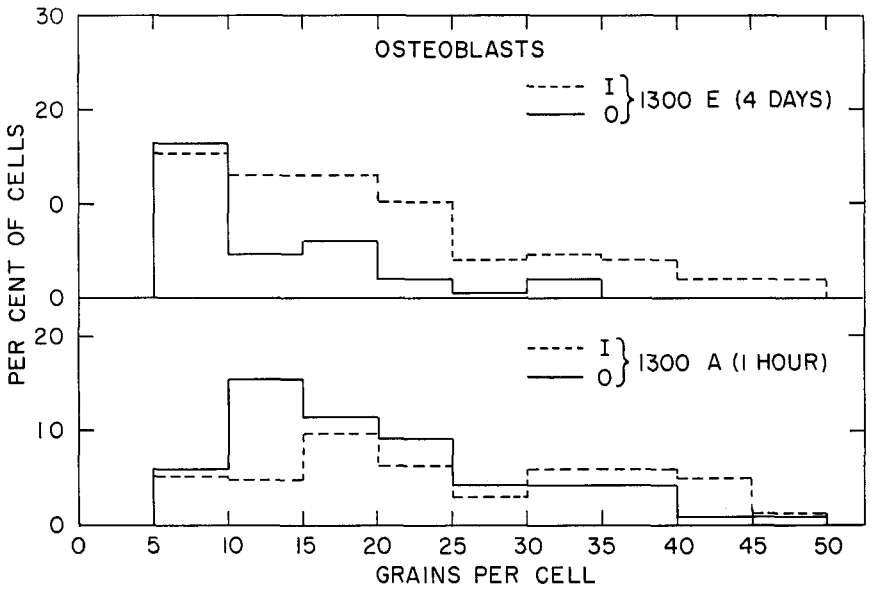

FIGURE 5 Grain count histograms for osteoblasts at 1 hour and 4 days after a single injection of tritiated thymidine, plotted for the two different regions, *I* and *O* (Fig. 2), of the bone surface. At 4 days the cells with higher grain counts predominate in region I; at 1 hour the distribution of grain counts in the two regions is similar.

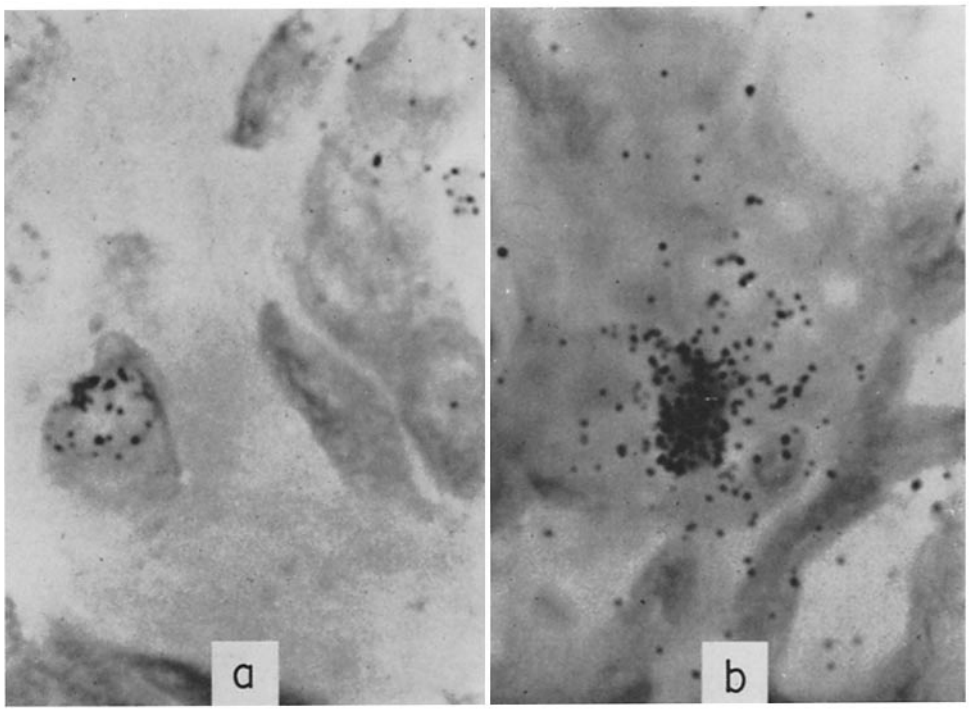

FIGURE 6 Autoradiograph showing nucleus labeled with tritium only (*a*) and with ^{14}carbon (*b*). × 1770.

from ^{14}C, the cells must be fairly well separated to allow a distinction to be made between the two types of autoradiographs. In the present material it was only possible to make measurements on the pre-osteoblasts and osteoblasts in the top halves of the loops or haversian canals (region *I*, Fig. 2). Here the labeled cells were far enough apart to prevent overlapping of their autoradiographs.

Provided the interval between the first injection and sacrifice of the animal is chosen so that few of the labeled cells will have divided during this time, then the length of the S period, T_S, is given by the following simple relation,

$$\frac{H}{C} = \frac{t_a}{T_S} \qquad (1)$$

where

$$\frac{H}{C} = \frac{\text{No. of cells labeled with } ^3H}{\text{No. of cells labeled with } ^{14}C + \text{No. of cells labeled with } (^{14}C + ^3H)}$$

and t_a is the time between injection of the first and second labels. In the present experiment, in the 3½ hour interval between injection and sacrifice there was evidence that some cell division had taken place among the pre-osteoblasts labeled with tritium only. In fact, out of 50 mitoses counted about 80 per cent were labeled. This indicates that the length of G_2 + prophase-metaphase, *i.e.* $t_{\gamma 2}$, is just greater than 3½ hours. T_S is then given by

$$\frac{H}{C} = \frac{t_a + (t_a - t_{\gamma 2} + t_b)}{T_S} \qquad (2)$$

where H/C, t_a, T_S, $t_{\gamma 2}$ are as already defined, and t_b is the time between injection of the second label and sacrifice of the animal (4).

A total of 850 pre-osteoblasts and 600 osteoblasts was counted. In the assumption that $t_{\gamma 2}$ = 3¾ hours, then by the use of equation (2) the *S* period of the pre-osteoblasts was found to be 6.2 ± 0.9 hours. For the osteoblasts which, it is assumed, do not divide, the *S* period was found from equation (1) to be 6.5 ± 1.6 hours. These results are in reasonably good agreement with those obtained by other authors for the dividing cells of the cartilage plate (1) and the mesenchymal cells of bone (2, 6, 7).

DISCUSSION

Cell Division

The cell cycle time[1] is the time taken for a cell to complete the generative cycle $G_1 + S + G_2 + M$, where G_1 is the presynthetic period, *S* is the period of DNA synthesis, G_2 is the postsynthetic period, and *M* is the time spent in mitosis. Following a single injection of tritiated thymidine, estimates of the length of the cell cycle and its different phases have been made for a variety of tissues by an analysis of the rate of appearance of labeled mitoses (8). In general, for mammalian cells, increase in the cell cycle time corresponds to a lengthening of G_1 whereas *S* and $G_2 + M$ have been found to vary relatively little (9), having values in the region of 6 to 10 hours and 1 to 5 hours, respectively. Recently, *S* and $G_2 + M$ have been measured for the dividing cells of bone in the metaphysis, periosteum, and endosteum of young rats, and have been found to be about 8 hours and 1 to 4 hours, respectively (2). Estimates of the *S* period for the cells in the present material, made by using the double-labeled thymidine technique of Wimber and Quastler (4), are in good agreement with the measurements mentioned above and other previous measurements for the cells in bone (1, 7). If similar values hold for $G_2 + M$ in the present material, then it is to be expected that all the labeled cells which are going to divide will have done so by 1 day after injection.

The evidence indicates that some cells take up thymidine and do not subsequently divide, at least by 3 or 4 days after injection. A possible explanation of this could be to assume that some of the labeled cells have a long $G_2 + M$ period and do not go through division until several days after injection. This possibility appears unlikely, as suggested above, on the grounds of the evidence so far for the length of the phases of the cell cycle in mammalian cells. Furthermore, if some cells have a long $G_2 + M$ period, then it would not be possible to attain 100 per cent labeled mitoses with time after injection, and, in the case of the young rat, the attainment of 100 per cent labeled mitoses

[1] The nomenclature used in these papers is that agreed upon at the Conference on Cell Proliferation in Dublin, August 1962, (see Analysis of Cell Population Kinetics by H. Quastler in the Proceedings of this Conference to be published by Blackwell, Oxford). Cell cycle time is equivalent to generation time previously used.

has certainly been found for the dividing cells of bone (2). On the other hand, it should be pointed out that neither the method of analysis of mitosis curves nor double labeling with thymidine, using a single time interval only, enables the detection of the presence of cells which take up thymidine and then do not divide.

The grain count spectra showed evidence that the number of labeled osteoblasts which had not instead of approximately ½ as expected. The grain count distribution at 1 day also showed evidence of cells with high grain counts, which could not have come from cells which had divided. This result has been confirmed with three different litters and it has been taken to indicate that a proportion of the pre-osteoblasts also take up thymidine and do not then proceed through cell division.

TABLE IV

Percentage of Cells with More than 25 Grains per Cell (Longitudinal Sections)

	Experimental results			Calculated values*			
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Rabbit (Time killed)	Pre-Osteoblasts	Osteoblasts	Pre-osteoblasts + osteoblasts	½ POb 0 Ob	¾ POb 0 Ob	All POb 0 Ob	All POb All Ob
13-00A (1 hour)	28.0	33.2	29.2				
13-00B (1 day)	10.9	14.9	12.5	14.1 (1.30)‡	8.3 (1.45)‡	3.8 (1.60)‡	1.0 (1.76)‡
13-00C (2 days)	7.0	16.5	10.8				
13-00D (3 days)	5.4	16.9	10.0				
1300-E (4 days)	3.8	18.5	10.0				

* Calculations were made for the pre-osteoblasts + osteoblasts, assuming various amounts of division of the pre-osteoblasts and osteoblasts, *e.g.* ½POb ≡ half of the pre-osteoblasts divide; 0 Ob ≡ none of the osteoblasts divide, etc.

‡ Calculated ratios of $\frac{\text{total labeled cells at time } t}{\text{total labeled cells at 1 hour}}$ to be compared with column (3), Table III.

divided by 3 or 4 days was of the same order of magnitude as the number initially labeled, and also that some cells had gone on to become osteocytes without division. The possibility, however, that there is *some* division of osteoblasts is not precluded, since some of these cells may be pre-osteoblasts which did not divide and which have since become osteoblasts. For, in the case of the pre-osteoblasts, there was also evidence that a fraction of the initially labeled cells did not divide within the first day after injection, even though these cells are considered to be the main site of cell proliferation (paper I). At one day after injection the mean grains per cell for the pre-osteoblasts had fallen to about ¾ of the value obtained at 1 hour

Non-Dividing Cells

In columns (1), (2), and (3) of Table IV the experimental results for the percentage of cells with more than 25 grains per cell (25 grains is approximately half the maximum grains per cell) are tabulated. In paper I it was noted that the number of labeled pre-osteoblasts per unit length of bone surface was about four times the number of labeled osteoblasts at 1 hour after injection. By using this fact and the results for 1300 A (1 hour), it is possible to calculate (a) the percentage of cells with more than 25 grains per cell, and (b) the total number of labeled cells to be expected after cell division for different conditions. If it is assumed that all labeled cells which divide have completed

their first division within 1 day after injection, then the calculation has been made assuming that different fractions of the pre-osteoblasts and osteoblasts divide (columns (4) to (7), Table IV). The figures in brackets are the calculated values for the ratio of the total number of labeled cells after division to the number at 1 hour, and these will be compared with the experimental results in Table III. Only 88 per cent of the cells which divide are seen after one division due to the fact that some of them will have less than 5 grains (our criterion for counting a cell as labeled), and this was allowed for in the calculation. The combined results for the pre-osteoblasts and osteoblasts were used so that it was not necessary to make any assumptions about the movement of cells from one compartment to another. No attempt was made to extend the calculation beyond one division of the labeled cells.

From a comparison of the experimental result in column (3), for the percentage of cells with more than 25 grains at 1 day after injection, with the calculated values in (6) and (7), Table IV, it can be seen that the data are *not* consistent with the assumption either that all the labeled pre-osteoblasts or that all the labeled cells divide. Comparison of column (3) with (4) and (5) shows that the experimental and calculated values are in closest agreement if it is assumed that all osteoblasts and between about 30 and 50 per cent of the pre-osteoblasts do not divide within the 1st day after injection.

The number of labeled cells per unit length of bone surface varied considerably for animals killed at the same time interval (see Table III), and within the various cell categories this quantity could differ by as much as a factor of two. It probably depends on the exact region of bone surface studied; our sampling was necessarily small. Nevertheless, the results for the ratio of the total number of labeled cells at 1 and 2 days after injection to 1 hour support the conclusion that 30 to 50 per cent of the pre-osteoblasts do not divide. The figures in column (3), Table III, for 1 and 2 days after injection agree better with the figures in brackets in columns (4) and (5), Table IV, than with those in (6) and (7).

It must be remembered, of course, that since our criterion for distinguishing pre-osteoblasts from osteoblasts is not very specific and is, in fact, largely a question of the position of the cell, then some cells designated pre-osteoblasts may well be osteoblasts and *vice versa*. Consequently, although the calculated values in Table IV, columns (4), (5), and (6), were obtained by assuming that there was no division of osteoblasts, similar values could have been obtained by assuming some division of osteoblasts and a correspondingly greater number of pre-osteoblasts which do not divide. With regard to this particular point it may be found easier to think of the pre-osteoblasts and osteoblasts as a single group of cells, a proportion of which take up thymidine after a single injection. Out of these labeled cells a certain number proceed through division within 1 day and therefore are part of the proliferating population in the normal way and are probably, without doubt, pre-osteoblasts. The rest of the labeled cells do not proceed to divide and may be either pre-osteoblasts or osteoblasts. In this connection it is of interest to note that labeled osteocytes were not seen before 3 days after injection (paper I). It is very likely that the osteoblasts labeled 1 hour after thymidine injections are "early osteoblasts," *i.e.* cells which have just differentiated into the functional state, and similarly the cells classified as pre-osteoblasts which do not divide may also be "early osteoblasts."

Finally, the question arises as to what is the explanation of the incorporation of thymidine, at the full rate associated with the doubling of DNA in preparation for division, into some osteoblasts and pre-osteoblasts which then do not divide. It is possible that cells which take up thymidine and do not divide may simply contain twice the normal diploid amount of DNA. This has not yet been checked. In this event, it may be that some cells having taken up thymidine differentiate into osteoblasts before having divided and then remain in this state while in the functional stage. Further investigations are in progress to determine whether the osteoblasts in this state do eventually divide after their main job of laying down matrix is finished. However, a proportion of the cells become osteocytes without going through division, and division of osteocytes (while they are osteocytes) is very unlikely.

Recently it has been pointed out by Pelc (10) that there is a considerable amount of evidence for the uptake of thymidine in highly differentiated cells which is not associated with cell division, and it is likely that the present results are supporting evidence for this. The osteoblasts are highly differentiated cells and, in growing regions such as those described here, they are active sites of RNA

metabolism and protein synthesis. The pre-osteoblasts are cells in varying stages of differentiation. Those which take up thymidine without subsequent cell division were to be found mostly within the haversian canals at the later time intervals and are likely, therefore, to have been the more highly differentiated of the pre-osteoblasts at the time of injection. It has been suggested (11, 12) that this uptake of thymidine in differentiated cells may be the renewal of DNA which is connected with cellular function and not with mitosis, though what, if any, this connection might be is not known. Another possibility, for which there is, as yet, no evidence, is that in these cells which do not divide the uptake of thymidine is associated with the manufacture of a polynucleotide such as polythymidylate. In any case, it is clear that the phenomenon of non-dividing cells requires further investigation.

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