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An autoradiographic study of the uptake of tritiated proline by osteoblasts during hibernation*

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Summary. Twenty-four LSH and LVG strain golden hamsters, *Mesocricetus* auratus, were used. Experimental animals were maintained at 5 C and allowed to hibernate. Control animals were kept at 27 C. Six animals (3 experimental, 3 control) were injected subcutaneously with 1 µCi of ^{3}H-proline/gm body wt. (Spec. act. 3 Ci/mM) after hibernation lasting 12 hours, 1 day, 3 days, or 7 days. Animals were killed 1 hour after injection and autoradiographs were prepared from 5 µm thick decalcified sections of femurs.

A greater number of endosteal cells were labeled than periosteal cells and also exhibited a greater magnitude of labeling throughout the study. Differences between endosteal and periosteal cells both in percentage of cells labeled and magnitude of labeling were maximum in control animals and progressively decreased with increasing periods of hibernation. A reduction in synthesis of matrix proteins during the early period of hibernation was seen and was attributed to a significant reduction both in average cell activity and in the number of active cells during hibernation. The latter phenomenon apparently made a large contribution to the reduced matrical synthesis. ^{3}H-proline uptake by osteoblasts probably retlects the reduced requirements of matrical synthesis during hibernation.

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Introduction

Several studies have demonstrated osteoporosis in jaws and long bones of hibernating mammals (Mayer and Bernick, 1958; Haller and Zimny, 1977; Steinberg et al., 1981). The loss of bone matrix during hibernation has been attributed mainly to osteocytic osteolysis (Whalen et al., 1972; Whalen, 1974; Steinberg et al., 1981). Vital dyes such as procion have been utilized to demonstrate protein-loss from bone during hibernation (Steinberg et al., 1979). However. the effects of hibernation on the svnthesis of bone matrix by osteoblasts has not been reported. Since proline serves as a precursor of collagen and is hydroxylated to hydroxyproline which is specifically incorporated into newly synthesized collagen, the availability of tritiated proline provides a tool for monitoring the activity of osteogenic cells during hibernation. The present study reports the uptake of ^{3}H-proline by osteogenic cells during hibernation and inactivity.

Materials and methods

Twenty-four adult male and female LSH and LVG strain golden hamsters (Mesocricetus auratus) were used. A control group of 12 animals was kept at 27 C and maintained on Purina Laboratory Chow and water a d libitum. An experimental group of 12 animals was placed in an environmental chamber, kept at 5 C, and allowed to hibernate. Purina Laboratory Chow and water were available in the chamber a d libitum. Experimental animals in 4 subgroups of 3 animals each, were injected

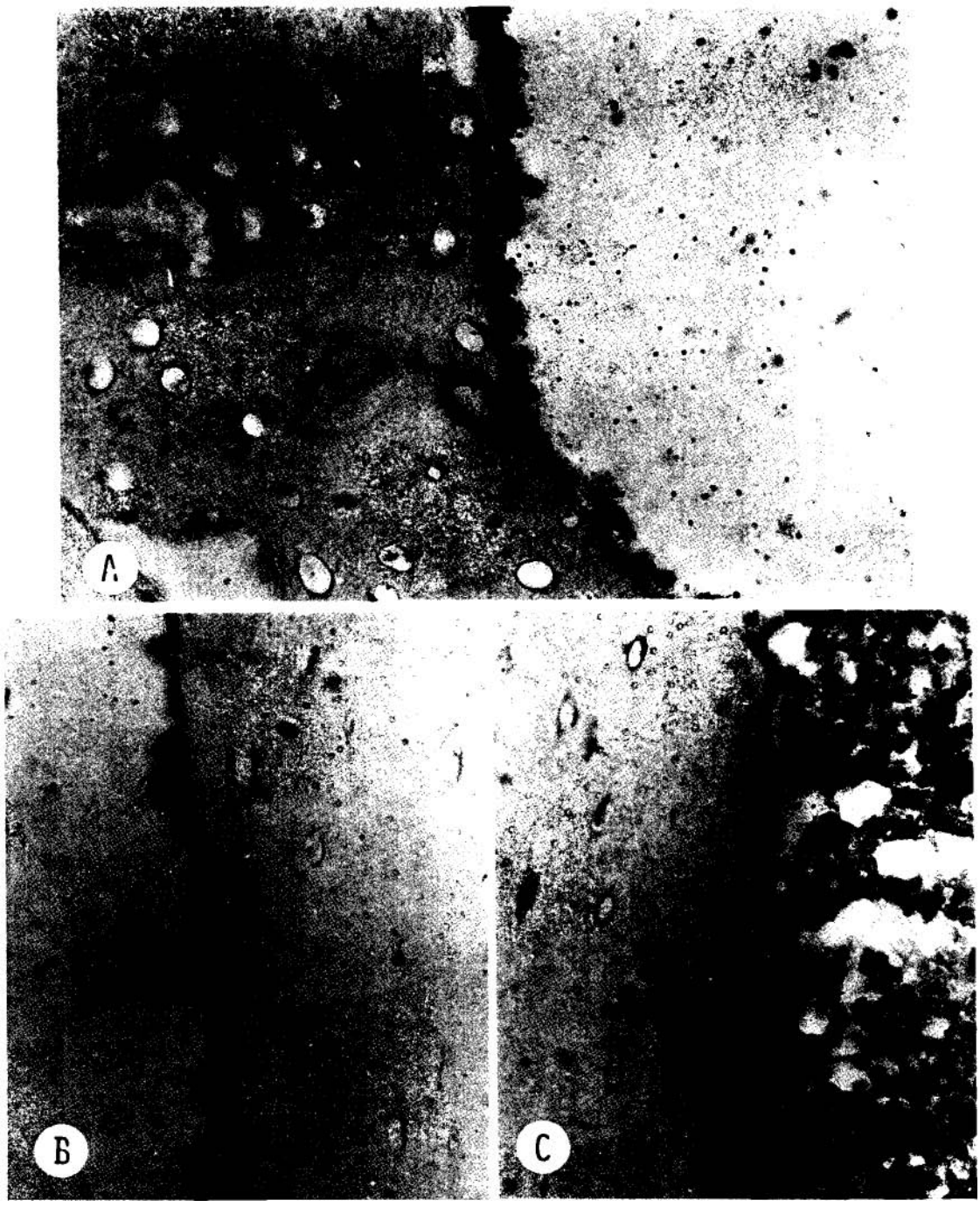

Fig. **1.** Autoradiographs of endosteal osteoblasts 1 hour after ^{3}H-proline administration (▪ 160). Control (A), 12 hours of hibernation (B), and 7 days of hibernation (C).

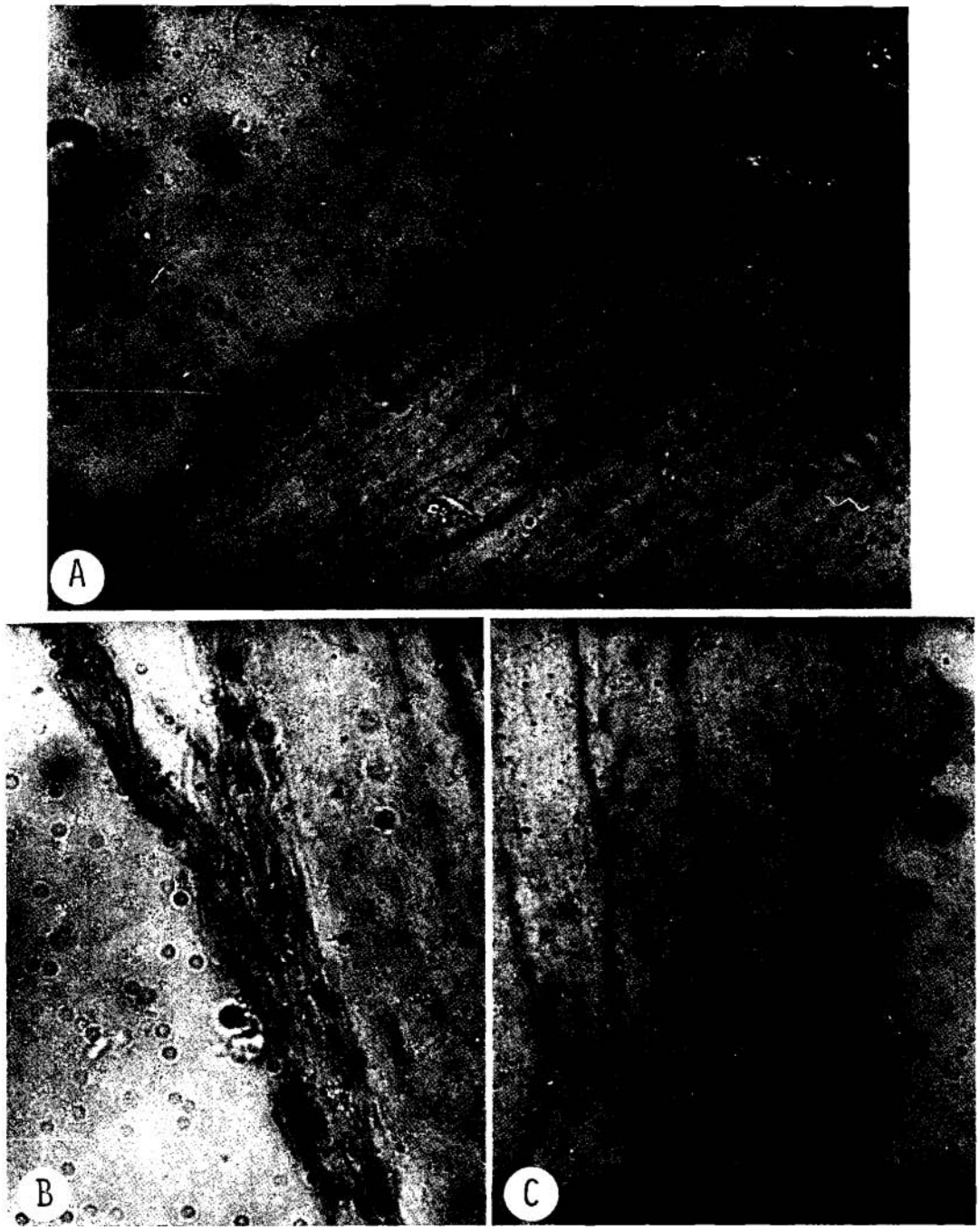

Fig. 2. Autoradiographs of periosteal osteoblasts 1 hour after ^{3}H-proline administration (× 160). Control (A), 12 hours of hibernation (B), and 7 days of hibernation (C).

Table. 1. Effect of hibernation on the percentage' of labeled osteoblasts 60 minutes after ^{3}H-proline administration

CELL COMPARTMENT	PERIOD OF HIBERNATION				
	Control	12 Hours	1 Day	3 Days	7 Days
Endosteal Ostoblasts	84.42 + 10.03	20.67 + 6.67	12.67 + 3.35	12.33 + 3.36	10.33 + 3.03
Periosteal Osteoblasts	45.08 + 7.83	12.33 + 3.83	10.00 + 2.45	7.00 + 2.23	6.67 + 1.87

1. Each value was calculated from an examination of 300 cells in each cell compartment from 3 animals per group (100 cells per animal).

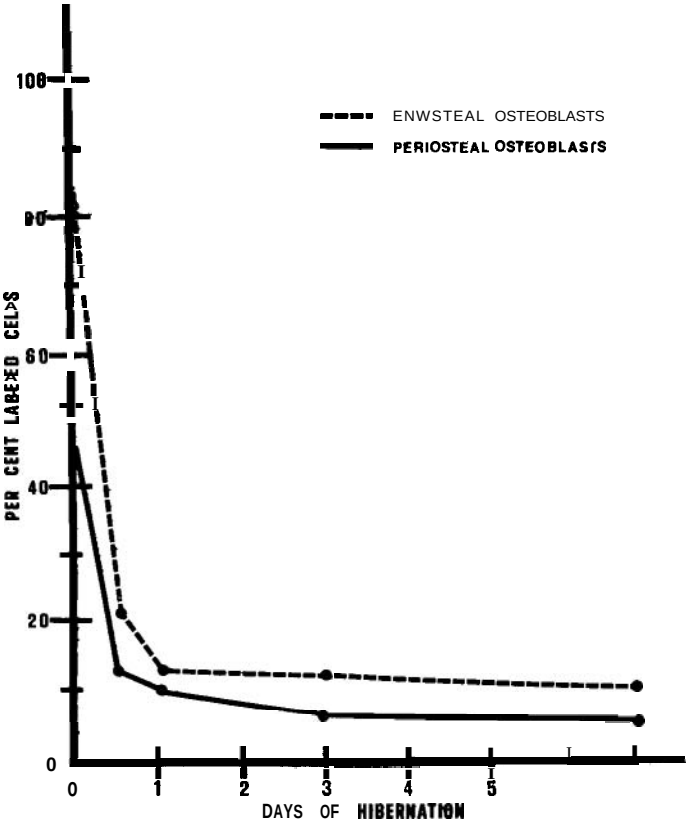

Fig. 3. Effect of hibernation on the percentage of 'H-proline labeled bone cells.

subcutaneously with lμCi of 'H-Proline (Schwarz-Mann Bioresearch, Orangeburg, N.Y.) (Specific activity 3Ci/mM) per gram body weight at hibernation periods of 12 hours, 1 day, 3 days, or 7 days. The control animals were also divided into 4 subgroups and similarly injected with 'H-proline. All animals were sacrificed one hour after 'H-proline administration. The femurs were removed and fixed in 10 per cent neutral buffered formalin, decalcified in Decal (Scientific Products), and embedded in Paraplast. Transverse sections, 5 μm thick, were cut at midshaft and three slides per specimen were prepared. Each slide contained 2 to 4 tissue sections.

The tissue sections were coated for autoradiography with Kodak NTB-3 liquid emulsion, exposed for 21 days at 4 C in a dry atmosphere, developed using D-19 developer for 3 minutes, fixed in Kodak sodium thiosulfate fixer and stained with hematoxylin. At × 1000, osteoblasts one to two cell layers deep at two cell compartments were examined. These consisted of (1) diaphyseal periosteal osteoblasts, and (2) endosteal osteoblasts. Two types of measurements were made from autoradiographs for each of the two bone cell compartments: (A) The percentage of labeled periosteal and endosteal osteoblasts was determined from a random examination of 100 cells in each cell compartment. A labeled cell was defined as one showing 3 or more autoradiographic grains. Cells with overlapping nuclear and/or cytoplasmic outlines were not included in the analyses. The average percentage of labeled cells in each cell compartment was calculated at each experimental period. (B) Average autoradiographic grain counts per cell for both endosteal and periosteal osteoblasts at each experimental period were determined. Two hundred labeled cells were examined per bone cell compartment for each animal and the number of reduced silver grains appearing over each cell was recorded. Again, cells with overlapping nuclear and/or cytoplasmic outlines were not included in the analyses and only cells with three grains or more were considered labeled. During grain counting, corrections for background were obtained by counting a total of three random fields per slide. The Student's t-test was used to examine differences among average grain counts for statistical significance ($p \leq 0.05$).

Results

The typical autoradiographic appearance of reduced silver grains over the nucleus and cytoplasm of endosteal osteoblasts in control and hibernating animals one hour after 'H-proline administration is shown in Figure 1. At a similar time period, the autoradiographic appearance of periosteal cells in control and hibernating animals is shown in Figure 2.

Percentage of labeled cells. After seven days of hibernation, the percentage of labeled osteoblasts decreased sharply from a control value of 84.42 per cent to a low value of 10.33 per cent in endosteal cells and

Table. 2. Average autoradiographic grain count[1] over osteoblasts in control and hibernating hamsters 60 minutes after ^{3}H-proline administration

CELL COMPARTMENT	PERIOD OF HIBERNATION				
	Control	12 Hours	1 Day	3 Days	7 Days
Endosteal Ostoblasts	8.38 + 0.94	4.21 + 0.59*	3.37 + 0.30*	3.14 + 0.14*	3.12 + 0.12*
Periosteal Osteoblasts	5.13 + 0.66	3.22 + 0.13*	3.21 + 0.03*	3.07 + 0.05*	3.03 + 0.01*

1. Each mean + standard deviation was calculated from at least 200 cells per animal from 3 animals per group.
* Significantly different from control value ($p \leq 0.05$).

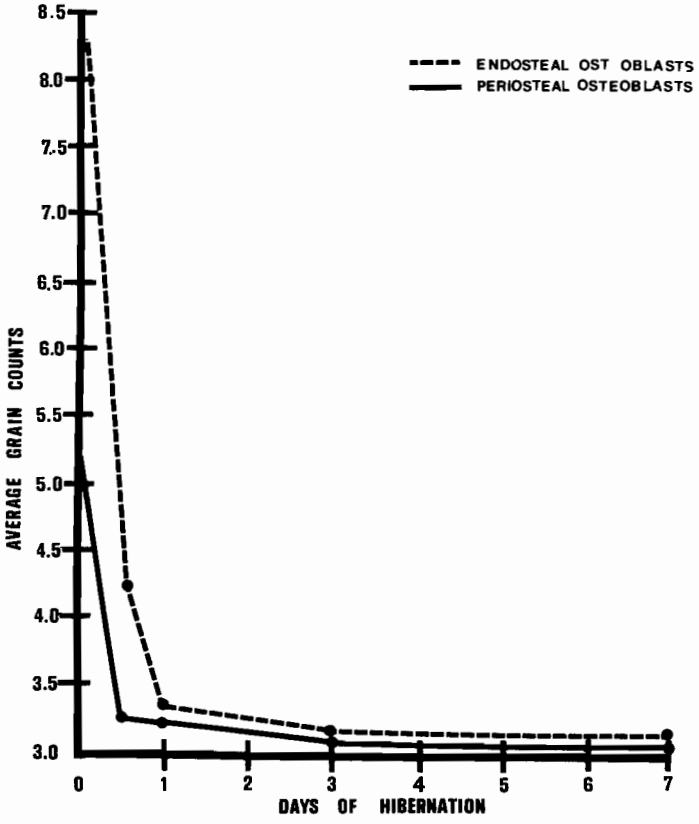

Fig. 4. Effect of hibernation on the average number of ^{3}H-proline autoradiographic grains over bone cells during hibernation.

from 45.08 per cent to 6.67 per cent in periosteal cells (Fig. 3, Table 1). The decrease was directly proportional to the duration of hibernation, the greatest reduction was found in the first 12 hours of hibernation. A more gradual decline in the percentage of labeled periosteal and endosteal osteoblasts occurred between 12 hours and 7 days of hibernation. Minimum values were noted at the end of the experimental period (7 days of hibernation). Note that (1) the pattern of decline was similar in the number of endosteal and periosteal osteoblasts actively incorporating tritiated proline during hibernation and (2) the endosteal compartment showed a higher percentage of labeled cells throughout the study.

Average Grain Counts: A. Endosteal osteoblasts - ^{3}H-proline uptake decreased sharply from an average control value of 8.38 grains to 3.12 grains per cell after 7 days of hibernation. The maximum rate of decline in grain counts occurred during the first 12 hours of hibernation with a reduction to approximately half of the control values. The decline in ^{3}H-proline uptake by endosteal osteoblasts was gradual but measurable in the period between 1 day and 7 days of hibernation (Fig. 4 and Table 2). B. Periosteal osteoblasts - ^{3}H-proline incorporation by periosteal osteoblasts decreased sharply from a high control value of 5.13 silver grains per cell to a low value of 3.03 silver grains per cell after 7 days of hibernation. The maximum rate of decline in grain counts occurred during the first 12 hours of hibernation with a reduction in average value to 3.22 silver grains per cell. The decline in isotope uptake was more gradual between the first and seventh days of hibernation (Fig. 4 and Table 2). Endosteal osteoblasts were significantly more active than periosteal cells particularly in control animals and during the early periods of hibernation. This difference was considerably reduced by 3 days of hibernation. In general, the pattern of ^{3}H-proline uptake was similar in the two cell compartments.

Discussion

In both control and hibernating animals the endosteal osteoblasts exhibited a higher level but a similar pattern of activity as compared to the periosteal osteoblasts. These findings are consistent with previous reports indicating that endosteal osteoblasts exceeded periosteal cell activity in all parameters thus far reported (Singh and Tonna, 1973, 1974; Tonna, 1976).

It has been previously shown that bone and cartilage cells decrease their matrical protein synthetic activity following growth and during aging thus exhibiting very few reduced silver grains intracellularly after administration of a tritiated precursor (Tonna, 1965). Other studies have also demonstrated the decline in precursor uptake by bone concomitant to the aging process

(Tonna, 1966, 1976; Singh and Tonna, 1974). Topographic analysis using ^{3}H-proline indicated that continued endosteal activity in older animals compensated for the structural thinning of the femoral shaft by bone production along the medullary canal (Tonna, 1976). These data are consistent with the low level of osteoblastic acitivity noted during hibernation in the present study.

Conventional ^{3}H-proline autoradiographic analyses examine average grain counts per cell. In the present study, the percentage of labeled cells after 3H-proline administrtion was also computed. It was reasoned that during hibernation two distinct and possibly independent mechanisms could account for the lower synthesis of matrix protein: the number of active cells could be reduced and/or the average activity per cell could be decreased. Since both the labeling index and average grains per cell were reduced, it appeared that, in both endosteal and periosteal cells, hibernation resulted in a decline both in the magnitude of cell activity and in the number of active cells. The maximum decrease occurred during the early period of hibernation (by 12 hours). During that early time period, the decrease in average grain counts was almost 50 per cent of control values; however, during the same time period, the percentage of labeled (active) cells showed the more dramatic reduction (to approximately 25 per cent of control values). Although during hibernation a significant decline in both the magnitude of osteogenic cell activity and in the number of active cells was responsible for the decrease in matrix synthesis, it appeared that the reduction in the number of active osteogenic cells had the more significant contribution. Whether this remains equally true during extended periods of hibernation or during aging remains to be investigated.

The dramatic reduction in labeling as a result of hibernation could perhaps be accounted for, in part, by concomitant changes in blood flow to bone with associated changes in extracellular proline compartments. The amount of label incorporating intracellularly into bone cells is dependent on the availability of the labeled amino acid to these cells. Precursor availability is related, at least in part, to the blood supply of bone. Previous studies have shown that there is a general reduction in blood circulation in the hibernating animal which most likely serves to prevent excessive heat loss (Matthews, 1956; Soivio, 1967). It has also been demonstrated that the blood circulation in the limbs of hibernating animals is so severly diminished that amputation of a limb during hibernation resulted in little or no blood loss (Cockrum, 1962). The decrease in blood supply to the limbs of hibernating animals would severly limit the availability of tritiated proline to the bone cells in these limbs and therefore decrease the amount of label incorporated. However, it is doubtful that the changes in microcirculation could alone account for a 75 percent reduction in the number of labeled cells as well as almost a 50 per cent reduction in the magnitude of ^{3}H-proline uptake per cell.

The decrease in osteoblastic activity could also account, in part, for the decrease in bone diameter and cortical thickness observed in long term hibernating animals (Steinberg et al., 1981). The present study demonstrated that only minimal ^{3}H-proline uptake was observed in both periosteal and endosteal osteoblasts after 1 to 3 days of hibernation. It is likely that this limited activity observed at the periosteal and endosteal borders was at least partially responsible for the maintenance of sufficient bone support required during arousal. Haller and Zimny (1977) reported osteocytic osteolysis during hibernation; the present study suggests that osteogenesis is adversely effected during hibernation. Our data are consistent with the reduction of osteogenesis reported during inactivity such as space travel (Morey and Baylink, 1978) as well as the reduced leukopoiesis by bone marrow during hibernation (Szilagyi and Senturia, 1972).

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