Calcium and Phosphate Levels in Bats (*Myotis lucifugus*) as Function of Season and Activity

Calcium and phosphorus are the major inorganic constituents of skeletal tissue, and are essential to varied functions of living systems. It has been known for some time that atrophy of bony tissue attends disuse of skeletal parts. Interest in mineral changes during immobility is currently strong, especially in the fields of mammalian hibernation and space medicine[1].

Materials and methods. In the present study a hibernating species, *Myotis lucifugus*, the little brown bat, was chosen for the following experiments: 1. animals in hibernation at 3 stages of the hibernating season and active summer bats were captured from their natural colonies and sacrificed for mineral analysis; 2. winter bats were kept at a high temperature to prevent hibernation, one group allowed to move freely and another group severely restricted in movement; 3. summer bats were divided into 2 groups and treated as in[2]. The experiments were designed to elucidate how hibernation affects the bone status of hibernating bats, and how relative activity versus relative inactivity influences the bones of winter and summer animals maintained under identical laboratory conditions.

Capture and care of experimental animals. Hibernating bats were obtained from a cave in southern Indiana, and were designated as being in: 1. 'early hibernation' (captured in November); 2. 'deep hibernation' (captured in February); or 3. 'late hibernation' (captured in April). In the laboratory they were housed in a moist gauze-lined metal container and kept in a cold (8 °C), darkened room until sacrifice.

Bats designated as 'winter free-flight' and 'winter restricted' were procured from their cave hibernaculum in the fall. The 'winter free-flight' bats were weighed, sexed, coded and then housed in a screened cage with 450 ft^3 of flying space; the cage was built inside a constant temperature room maintained at 35 °C (95 °F) with a relative humidity of approximately 20% (Figure). To ensure that the bats got sufficient food, they were trained to eat mealworms (*Tenebrio molitor* larvae) from dishes in individual cages, and were placed in these cages daily to feed; afterward the cages were opened and the bats were free to fly again. Water was provided ad libitum in the individual cages and in numerous dishes on the floor of the free-flight room. The 'winter restricted' bats were housed in the same room, but their movements were curtailed by keeping them in a confined space of 100 in^3 (Figure inset). The restricted bats also were fed mealworms and were given water ad libitum.

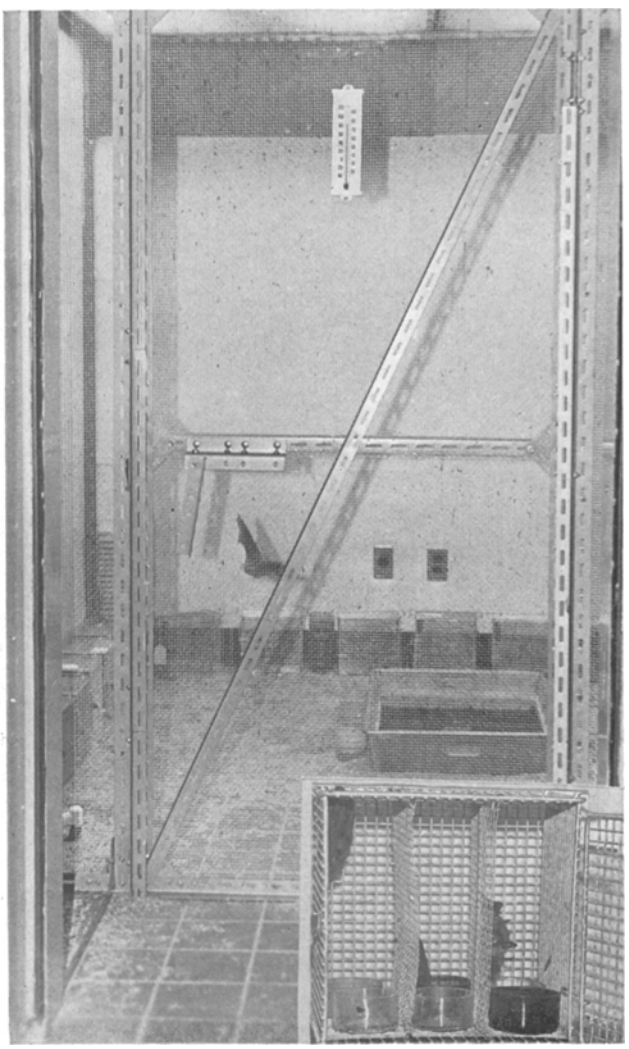
'Summer' bats were collected from an attic colony in June and were sacrificed immediately. Animals designated as 'summer free-flight' and 'summer restricted' were obtained from the colony and housed in the same manner as the 'winter free-flight' and 'winter restricted' groups.

After a chronic period in the free-flight room or restraining cage (approximately 60 days), the animals were sacrificed by decapitation and blood, bone, urine, and fecal samples were retained for analysis.

Analytical methods. Plasma calcium. Most of the calcium of the blood is in the plasma. Therefore, following sacrifice by decapitation, each animal was exsanguinated and the whole blood promptly drawn into calibrated (volume = $\pi r^2 h$) microhematocrit capillary tubes[2] and centrifuged at 11,500 rpm for several min. The plasma fraction was processed for routine analysis by atomic absorption spectrophotometry[3]. Protein was precipitated with 10% TCA. A working curve for calcium was graphed by ordinating absorbance of standard calcium solutions with concentration. The concentration of calcium in plasma samples was read from the curve, multiplied by the sample dilution factor, and expressed as mEq/l.

Bone sample preparation. The bones supporting the wing membranes of the bat were analyzed for mineral content. Muscle and extraneous connective tissues were carefully removed, and the bones were weighed and placed in a drying oven overnight. After their dry weight was determined, they were placed in a crucible, ashed in a muffle furnace, and their ash weight recorded.

Bone and fecal calcium. A known weight of bone ash was put into solution for atomic absorption spectrophotometric analysis using a modification of the procedure described in *Analytical Methods*[3]. Quantification was accomplished by consulting the standard curve, and was expressed as mEq calcium/kg bone ash. A fecal sample was obtained at the time of sacrifice, and was treated in the same manner as the bone sample.



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[2] G. M. Guest and V. E. Siler, J. Lab. clin. Med. *19*, 757 (1934).
[3] *Analytical Methods for Atomic Absorption Spectrophotometry* (Perkin-Elmer Corp., Norwalk, Conn. 1966).

Plasma, urine, and bone phosphate. The concentration of inorganic phosphate in plasma, urine and bone samples was determined with a Beckman Model B photoelectric spectrophotometer at a wavelength of 660 nm, using a modification of the method of FISKE and SUBBAROW[4]. Quantification was achieved by constructing a standard curve for phosphate and comparing absorbance readings of the unknowns to it. Plasma and urine phosphate concentrations were expressed as mEq/l and that of bone as mEq/kg.

Microscopic examination of bone. A small section of bone from the epiphyseal end of the right humerus of each bat was retained for histological examination. Following decalcification, the segments were sectioned at 7 μ and regressively stained with hematoxylin-eosin. The sections were examined microscopically for osteoclasts, and the results expressed quantitatively as number of osteoclasts/100 7-μ sections from bats of a given treatment (e.g. early hibernation).

Results. The concentrations of inorganic calcium and phosphate detected in the plasma, bones, and excreta of *M. lucifugus* are summarized in the Table. It can be seen that the mineral content of bone decreases as hibernation progresses, and begins to return to higher levels in the summer animals. A consistent pattern of mineral changes in plasma is not evident in the hibernating animals, although calcium levels appear to be highest in early hibernation (see Table). No uniform effects on bone mineral concentration are apparent in free-flight and restricted bats. Bone calcium concentration is higher in winter free-flight bats than in winter restricted animals, but the reverse is true for summer free-flight and restricted bats. The phosphate content of bone is greater in winter free-flight bats than it is in winter restricted bats, but the opposite is true for summer free-flight and summer restricted animals. The plasma picture is consistent in showing higher concentrations of both calcium and phosphate in restricted bats as compared with their respective free-flight counterparts (see Table).

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Calcium and phosphate concentrations, and osteoclast counts in *Myotis lucifugus* as function of season and activity

	Calcium								
	Plasma[a]			Bone[b]			Feces[b]		
	$\bar{X}$	S.D.	*t*-value	$\bar{X}$	S.D.	*t*-value	$\bar{X}$	S.D.	*t*-value
Early hibernation	12.1 ± 3.1			22,313 ± 1429			5908 ± 1689		
			> 3.453[f]			> 3.609[f]			> 1.536
Deep hibernation	8.5 ± 1.5			19,734 ± 1923			7412 ± 2596		
			> 0.536			> 3.542[e]			> 5.197[e]
Late hibernation	8.8 ± 1.2			16,656 ± 1964			2774 ± 1109		
			> 2.472[d]			> 1.982			> 2.798[d]
Summer	7.5 ± 1.1			18,073 ± 1122			5371 ± 2719		
Winter free-flight	8.6 ± 2.4			20,303 ± 2081			4658 ± 1931		
			> 0.276			> 1.460			> 0.312
Winter restricted	9.9 ± 3.7			19,300 ± 625			4923 ± 1853		
Summer free-flight	5.6 ± 0.7			18,005 ± 2447			4472 ± 1842		
			> 2.016			> 1.726			> 1.548
Summer restricted	6.3 ± 0.6			19,989 ± 2688			6000 ± 2318		

	Phosphate									Osteoclasts[c]
	Plasma[a]			Bone[b]			Urine[a]			No./100 7-μ sections
	$\bar{X}$	S.D.	*t*-value	$\bar{X}$	S.D.	*t*-value	$\bar{X}$	S.D.	*t*-value	
Early hibernation	0.6 ± 1.4			23,123 ± 2667			2.6 ± 3.3			4
			> 0.065			> 0.902			> 1.270	
Deep hibernation	0.7 ± 1.4			22,246 ± 1768			5.5 ± 3.3			5
			> 0.007			> 2.463[d]			> 1.578	
Late hibernation	0.7 ± 0.8			20,705 ± 888			13.3 ± 11.2			9
			> 2.219			> 0.977			> 0.632	
Summer	2.5 ± 2.4			21,105 ± 941			8.7 ± 6.6			1
Winter free-flight	1.5 ± 2.3			25,661 ± 1512			6.7 ± 4.1			3
			> 1.230			> 3.857[e]			> 1.282	
Winter restricted	4.5 ± 7.2			23,547 ± 849			8.5 ± 4.6			3
Summer free-flight	6.5 ± 3.3			20,720 ± 1280			10.1 ± 3.7			4
			> 1.441			> 0.013			> 0.385	
Summer restricted	10.2 ± 7.5			20,727 ± 1261			8.9 ± 9.8			7

[a] Values expressed in mEq/l. [b] Values expressed in mEq/kg. [c] Count made on proximal epiphysis end of right humerus. [d] Significant at 0.05 α: $t_{0.05}$ (18) = 2.101. [e] Significant at 0.01 α: $t_{0.01}$ (18) = 2.878. [f] Significant at 0.01 α: $t_{0.01}$ (20) = 2.845.

Fecal calcium is greater in earlier stages of hibernation than near spring arousal (see Table), but returns to higher levels in summer bats, reflecting active feeding again by the latter group. More calcium appears in the feces of restricted bats than in the excreta of free-flight bats. The phosphate concentration of urine increases with length of hibernation, dropping to lower levels in summer bats. Free-flight bats in winter tend to excrete less phosphate in their urine, according to these data, than do winter restricted bats; however, the reverse results are observed in summer laboratory animals. The Table presents *t*-values for differences between mineral concentrations in seasonal and laboratory bats.

An examination of the Table also shows that the osteoclast count is increased with the lengthening of hibernation, and returns to a low level in summer. Osteoclasts are more numerous in summer restricted bats than in summer free-flight bats, and appear to be equally evident in winter free-flight and winter restricted bats.

Discussion. The results of this study affirm that the bones of a hibernating or inactive animal may be affected significantly by the lack of movement of skeletal members and by the diminishment of muscular forces which act on the bones when the animal is active. The concentration of calcium and phosphate in the bones of the little brown bat is lower at successive stages of hibernation (see Table). Some of the differences are statistically significant at 0.01 or 0.05 α. The loss of mineral from bone is reflected by increased losses of phosphate in the urine during hibernation, and by the appearance of higher amounts of fecal calcium in deep hibernation. The fecal calcium picture is partially confounded, however, by the fact that significant amounts of fecal calcium are of dietary (i.e. gastrointestinal) origin.

Diet undoubtedly influences mineral loss from bone, in addition to immobility. The body's store of supplies necessary for homeostasis is tapped during periods of curtailed feeding, as during hibernation; however, metabolism during hibernation is minimal and great quantities of energy sources are not rapidly utilized. Results of this study indicate that immobility may supersede diet as a causative factor in bone demineralization. Bone levels of calcium and phosphate are lower in winter restricted bats than in winter free-flight bats, although both groups received identical diets. Bone phosphate especially seems to be affected, showing a difference between winter free-flight and winter restricted animals that is significant at an α level of 0.01 (Table). Fecal calcium is also higher in the winter restricted than in the winter free-flight group, suggesting greater calcium losses in the immobilized animals and confirming the results of bone analyses in the 2 groups.

The findings of the histological study in general support the biochemical results. For example, in the hibernating bats, as bone mineral content diminishes, the osteoclast number increases steadily (Table). In the June (summer) bats bone mineral concentrations are on the rise, and the osteoclast count has fallen off from 9/100 sections during late hibernation to 1/100 sections in the summer animals. This is evidence to strengthen the view that osteoclasts are fundamentally involved in bone resorption.

When considering the bone minerals in *summer* free-flight and restricted bats, some apparent inconsistencies arise. For example, bone calcium levels are higher in the restricted bats. Bone phosphate concentrations appear to be approximately equal in both groups. The fact that bone minerals in summer laboratory bats are not lower in the restricted group may indicate the involvement of another component of bone resorptive processes, the endocrine system. It is well established that parathormone influences bone resorption, and GAILLARD's[5] studies indicate that it does so by stimulating osteoclastic development and activity. KAYSER and FRANK[6] suggest a relationship between osteoporosis in hibernating European hamsters and the known functional hyperactivity[7] of the parathyroids in this species during the months of hibernation. It is conceivable that a similar cycling of activity occurs in the parathyroids of *M. lucifugus*, so that in the winter months normal functional hyperactivity of these glands was further augmented by relative immobility of skeletal members in the restricted bats, thereby leading to a greater loss of bone mineral in that group than in the winter free-flight group. The summer restricted bats may not have displayed the same bone demineralization because of the recession of parathyroid activity following arousal from hibernation.

No overall pattern is evident in the results of mineral analyses of bat plasma. It does seem apparent, however, that calcium levels are somewhat greater during hibernation than during the summer active season (Table). Indications are that there is also more plasma calcium in restricted bats than in those able to fly and move about freely. These results are in general agreement with those obtained in the hibernating marmot[8], in the hedgehog[9], and in the hamster[10–12]. Hibernating bats in the present study display a distinct hypophosphatemia (although there are large standard deviations for these data) as well as hypercalcemia. This is further evidence that the parathyroid glands may be hyperactive during hibernation, for 2 of the distinct clinical manifestations of hyperparathyroidism are increased plasma calcium and low plasma phosphate[13,14].

Hibernation and enforced immobility seem to cause bone demineralization in *M. lucifugus*. Further studies are indicated to determine the role of the endocrine system in demineralization and the precise mechanisms involved.

Zusammenfassung. Untersuchungen an Laboratoriums-Fledermäusen *Myotis lucifugus* während aller Phasen der Winterschlaflethargie zeigen, dass Unbeweglichkeit der Tiere entscheidend auf den Verlust an Knochenmineralien wirkt. Die Vermehrung an Osteoklasten sowie die Hyperkalzemie und die Hypophosphatemie bei winterschlafenden Fledermäusen weisen ebenfalls in Richtung der Resorptionsvorgänge im Knochen.

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