PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 176, 342–345 (1984)

Mineralization in Rat Metaphyseal Bone Exhibits a Circadian Stage Dependency[1] (41880)

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Abstract. Density gradient fractionation analysis of rat metaphyseal bone was used to delineate the biorhythmic changes in bone matrix mineralization. Seventy-two 4-week-old rats were entrained to 12-hr light, 12-hr dark cycles (light, 0800–2000 hr; darkness, 2000–0800 hr) for 4 weeks. All animals were fed *ad lib.* on Purina laboratory rat chow and tap water. Groups of 10–12 rats were killed by cervical dislocation at 4-hr intervals during a 24-hr period, and the tibias were then biopsied and frozen in liquid N_2. Metaphyseal bone was fractionated via bromoform–toluene density gradients into specific gravity fractions ranging from 1.7 to 2.8. Density gradient fractions were analyzed for concentrations of calcium and inorganic phosphorus.

Chronograms indicated that the accumulation of both calcium and inorganic phosphorus into the newly forming/least-dense mineral moieties of bone (1.3–1.7 sp grav) showed a single peak in the biorhythm of the rat. A statistically significant circadian rhythm of mineralization was detected for calcium ($P < 0.001$) and inorganic phosphorus ($P < 0.039$), with peaks during the environmental dark span. These results suggest that the physiological phasing of bone mineralization in the light–dark synchronized rat, is similar to that previously noted for cartilage mineralization and is antiphasal to the midday peak in bone collagen synthesis.

Circadian rhythms are a characteristic feature of the physiology of most tissues in the body. In a general sense, this means that physiological functions have excursions in activity that often range from two- to fourfold, with a single peak (acrophase) and minimum (nadir) during the course of a 24-hr day (if sampling is performed at 3- to 4-hr intervals). There is a growing awareness that these biorhythms are not only intrinsically interesting, but also can have a practical impact on physiological/endocrinological investigations (1, 2).

Our laboratory has focused its efforts on the chronobiology of the skeleton and has examined a spectrum of normal metabolic rhythms in bone and cartilage (3–5). Most recently we have asked how the timing of these changes is related to the timing of mineralization in rat epiphyseal cartilage (4), and the data suggested that the mineralization of the epiphyseal cartilage occurs most actively at night. Since this mineralization of cartilage is antiphasal to the rhythm of net collagen synthesis in both epiphyseal cartilage (3) and bone (5), and since a lag-time between matrix formation and mineralization has been demonstrated in bone (6, 7) as well as cartilage (4), this study was undertaken to more fully delineate the biorhythmic changes in bone matrix mineralization.

Materials and Methods. During the spring months, 72 young male rats (Sprague–Dawley; 4 weeks of age; 100–120 g body wt) were conditioned to a 12-hr/12-hr LD cycle for 4 weeks (light, 0800–2000 hr; darkness, 2000–0800 hr). All animals were fed Purina laboratory rat chow *ad lib.* and tap water. The animals (275 ± 25 g body wt) were then randomized into groups of 10–12, and a different group was killed (cervical dislocation) every 4 hr throughout a single 24-hr time span. The tibias were recovered at autopsy, frozen in liquid N_2, lyophilized, and stored at −80°C. The proximal metaphyseal bone trabecular region was dissected from each tibia at the time of use.

Mineral maturation profiles. The status of metaphyseal bone matrix mineralization was assessed by a bromoform–toluene density gradient fractionation technique as previously used in this (8, 9) and other laboratories (10, 11). The bone was ground to a 40 μm particle size in a Spex Mill, and separated into four

[1] Work supported by USPHS Grant AM16391.

0037-9727/84 $1.50

specific gravity fractions (1.3–1.7, 1.8–1.9, 2.0–2.1, and 2.2–2.8 sp grav). The amount of calcium (Ca) or inorganic phosphorus (P_i) in each specific gravity fraction was analyzed by methods previously described (8), and the data were expressed as a percentage of the total Ca and P_i. It has already been established that this gradient profile of bone reflects states of mineral–collagen maturation/mineralization (8, 10, 12). The lowest density, least mature fraction (1.3–1.7) is synonymous with newly mineralizing moieties of bone (10, 12). Specific gravity fractions above 1.7 are not correlated with the newly forming bone mineral.

Statistics. The presence or absence of a significant circadian rhythm was assessed by the Cosinor method of Halberg (13). This is a least-squares analysis using a cosine function and is usually fitted to a 24-hr time interval. The test provides a P value which indicates the significance of the fit of the cosine curve to the data, and thus it is a way to detect the presence of statistically significant rhythms. The analyses also provide information about the time in the rhythm when the peak value in the curve (acrophase) would be expected to occur.

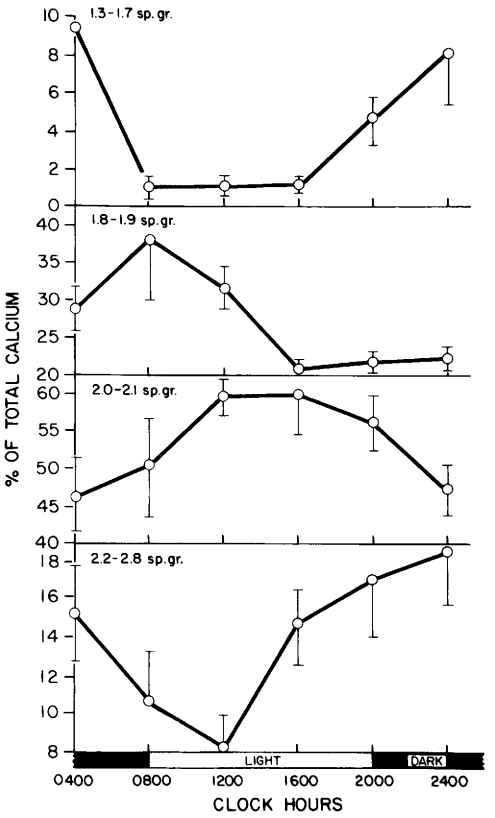

FIG. 1. Plot of the circadian changes in rat metaphyseal calcium: the bone was fractionated into four specific gravity fractions on bromoform–toluene density gradients as described (8). $N = 6$ for each point of observation, and each point represents the mean ± SEM. The statistical significance of each of the rhythms and their acrophase times are reported in Table I.

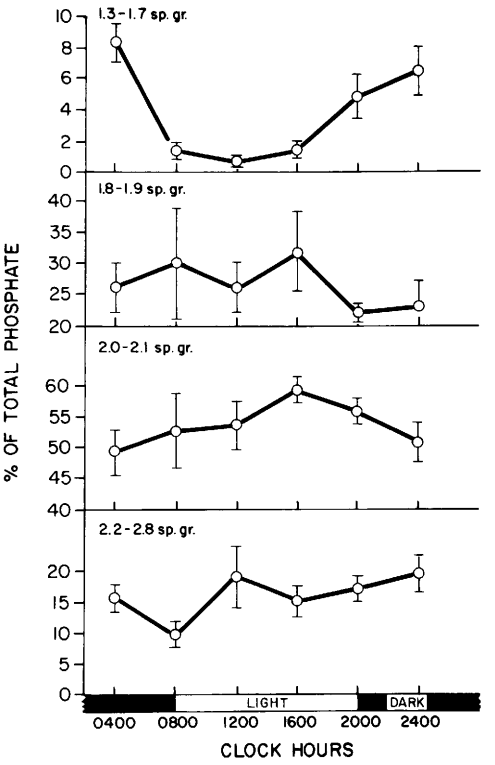

FIG. 2. Plot of the circadian changes in rat metaphyseal inorganic phosphorus: the bone was fractionated into four specific gravity fractions on bromoform–toluene density gradients as described (8). $N = 6$ for each point of observation, and each point represents the mean ± SEM. A statistically significant rhythm as defined by the cosinor method was only found for the 1.7-sp grav fraction (see Table I).

Results. Density gradient analysis of the rat tibial metaphyseal bone from rats revealed that there were striking changes in the mineral profiles at different times in the 24-hr body rhythm (Figs. 1 and 2). In normal 8-week-old male rats, the percentage of the total bone mineral in the lowest density gradient fraction (1.3–1.7 sp grav) was highest during the daily

TABLE I. COSINOR ANALYSIS OF THE BIORHYTHMICITY OF BONE CALCIUM AND INORGANIC PHOSPHORUS LEVELS: CHARACTERIZATION VIA DENSITY GRADIENT FRACTIONATION

Measurement	Density gradient fraction (sp grav)	P^a	N	Peak or acrophase in clock hours (±95% CL)
Calcium (% of total)	1.7	0.001	36	1.18 ± 0.86
	1.8–1.9	0.010	33	8.13 ± 1.16
	2.0–2.1	0.020	35	15.08 ± 1.28
	2.2–2.8	0.009	34	22.74 ± 1.13
Inorganic phosphorus (% of total)	1.7	0.039	36	24.44 ± 1.40
	1.8–1.9	NS	36	—
	2.0–2.1	NS	36	—
	2.2–2.8	NS	36	—

[a] P value was determined according to the Cosinor statistical analysis as described under Materials and Methods. In the absence of a significant fit of the data to a cosine curve, no peak or acrophase time can be ascribed to the data.

dark span when it accounted for as much as 8–10% of the total bone calcium or inorganic phosphorus. The 1.3- to 1.7-sp grav fraction accounted for only 1.0% of total bone Ca and P_i during the daily 12-hr light span. Cosinor analysis of the data (Table I) revealed that the distribution of Ca and P_i within the 1.3- to 1.7-sp grav fraction did show a significant circadian rhythmicity. The peak time (acrophases) of Ca accumulation in this low-density fraction was 0100 hr, while that for P_i was 2400 hr. These changes in the mineral distribution throughout the 24-hr period were not accompanied by alterations in either total bone Ca or P_i (data not shown). The average metaphyseal calcium level was 186.03 ± 3.54 mg/g dry wt of bone and inorganic phosphorus was 95.52 ± 2.93 mg/g dry wt of bone.

The profiles in Figs. 1 and 2 also suggest a time-related shift of Ca and P_i into progressively higher specific gravity (more dense/mature mineral) fractions. The variations within each clock-hour group were less for bone Ca than P_i (see P values for different specific gravity fractions in Table I).

Discussion. The intent of this study was to extend our observations about the normal biorhythmic changes of rat tibial bone metabolism. Previously, we and others have demonstrated that in light–dark (LD) synchronized *ad lib.*-fed rats, the levels of bone and cartilage matrix production peak during the early hours of the light span (daytime period) (3, 5, 14). In such animals, epiphyseal cartilage mineralization appeared to progress most actively during the environmental dark span (nighttime), but we had no firm information about the time in the body rhythm when the rat tibiae mineralized. However, given the synchrony of matrix biosynthesis in cartilage and bone and the fact that a mineralization lag time has been documented in both tissues (3, 4, 6, 7), it seemed reasonable to anticipate that the most active period of new bone mineralization would occur at night. The data developed in this study by the density gradient fractionation technique affirmed that inference. The mineral maturation profiles showed that the highest percentage of newly deposited mineral (1.3–1.7 sp grav) occurred during the nocturnal hours of the 24-hr period. Thus, it appears that the pace of cartilage and bone mineralization is antiphasal to the pace of matrix biosynthesis in the *ad lib.*-fed, LD-synchronized rat (4, 5).

While the density gradient fractionation method has provided valuable information about how metabolic disorders of bone affect mineral maturation (8, 9, 15–18), this study demonstrates for the first time that the precise profiles are circadian stage-dependent. The shifts in mineralization profiles across the 24-hr time span call attention to the need to observe basic chronobiological principles in experimental design and interpretation of laboratory results.

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Received January 23, 1984. P.S.E.B.M. 1984, Vol. 176.
Accepted April 10, 1984.