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Adenosine nucleotides in rat bone measured by ion-pair reversed-phase high-performance liquid chromatography: effect of hemorrhagic shock, with and without retransfusion of blood

M. Manoharan, R.M. Schwille, P.O. Schwille*

Division of Experimental Surgery and Endocrine Research Laboratory, Department of Surgery, University of Erlangen, Postfach 3560, 91054 Erlangen, Germany

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Abstract

The measurement of bone adenosine nucleotides (ATP, ADP, AMP) using a simple HPLC procedure is described for rat tibia; the response to hemorrhagic shock with and without blood retransfusion is also described. With respect to the measurement of nucleotides, a number of validation criteria are met. In the anesthetized intact rat (Normal) there was a declining gradient of the three nucleotides, expressed as nmol per g dry matter, from proximal over middle to distal diaphysis, with the mean ratio ATP/ADP (0.21, 0.20, 0.20) and the mean energy charge (0.34, 0.31, 0.30) being low. Irrespective of the anatomic site, hemorrhagic shock of 30-min duration evoked a further decrease versus Normal of ATP, ATP/ADP and energy charge. Blood retransfusion after shock kept nucleotides and other variables in the proximal and distal, but not the middle, diaphysis within normal limits. It was concluded that: (i) bone nucleotides are reliably measurable by HPLC, allowing the described method to be recommended for wider use in bone research and related areas; (ii) in contrast to more parenchymatous tissues, low ATP, ATP/ADP and energy charge may be characteristic for long bones, pointing towards different energy metabolism; and (iii) bone is a ''shock organ'', reflecting blood hypoperfusion, O_2 deficiency and decreased ATP in this situation.

Keywords: Hemorrhagic shock; Blood retransfusion; Adenosine nucleotides; Nucleotides

1. Introduction

In intermediary metabolism cellular energy is stored in the form of nucleotides, such as adenosine-triphosphate (ATP). During periods of insufficient oxygen supply the latter is degraded to adenosine-diphosphate (ADP) and adenosine-monophosphate (AMP). A knowledge of the state of tissue energy stores therefore provides insight into numerous physiological and pathophysiological processes at the organ level, e.g. during low systemic blood flow, local ischemia, and hypoxia. Over the past decades, numerous methods for the measurement of nucleotides in biological samples, tissues included, have been described, most of them utilizing specific enzymes and spectrophotometric kinetic analysis [1]. Such methods are sensitive and can be performed with standard equipment in many laboratories. However, high-performance liquid chromatography

*Corresponding author. Present address: Department of Surgery, University Hospital, Maximiliansplatz 2, D-91054 Erlangen, Germany. Tel.: +49-9131-59790; fax: +49-9131-533-331.

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(HPLC), although requiring more specialized equipment, is now increasingly used, probably due to its equal specificity but more favorable cost/benefit ratio.

To separate the nucleotides ATP, ADP and AMP, most HPLC methods require salt gradients with high final molarities, rendering the procedure more complex. Information on nucleotides in hard tissues, such as bone, appears scanty, and we were unable to identify publications describing the use of HPLC. Using the bioluminescent assay, Yamaguchi et al. found ATP in amounts ranging from 2 to 10 nmol/g wet tissue in rat femur diaphysis [2], while Leung et al. found 2 nmol/g wet tissue ATP in the tibial diaphysis after prior perchloric acid extraction [3]. Interestingly, a general feature of most existing methods for the quantitation of ATP appears to be the lack of validation of the analytical method in use. Thus, in our hands, perchloric acid treatment of samples at 4°C yields falsely low ATP, and a rapid time-dependent decline in ATP and increase in ADP and AMP appear characteristic for methods using chilled but not deep-frozen samples.

During the course of studies on bone microcirculation under various conditions, we became interested in the state of bone nucleotides and energy stores. Such information is of importance not only for physiology but also for the diagnosis, treatment and prognosis of patients with fractures of long bones, especially when these occur in combination with disturbed blood supply and increasing age [2–4]. Below we report on a simple isocratic HPLC method for the determination of adenosine nucleotides in normal rat tibia, and their response to shock and to shock treatment.

2. Materials and methods

2.1. Instrumentation

The HPLC system consisted of the Jasco 880 PU Intelligent pump (Jasco Labor- und Datentechnik, Groß-Umstadt, Germany); Rheodyne valve, with 10-μl sample loop and valve operated with electronic valve actuator (Model 732, Bischoff Analysentechnik, Leonberg; Germany); Ultrasphere ODS (5 μm), 250×4 mm I.D. (Beckman, Munich, Germany); Jasco 870-UV Spectrophotometer (Jasco Labor- und Datentechnik, Groß-Umstadt, Germany); Apex computer integration system with Autochrom interface (ESWE, Sinsheim, Germany); and cell sonicator (Novodirect, Kehl, Germany).

2.2. Chemicals

Only deionized double-distilled water and analytical grade substances were used. Adenosine (tri-, di-, monophosphate) nucleotides, herein named ATP, ADP, AMP, were supplied by Sigma, Deisenhofen, Germany, and used as standards. Anhydrous di-potassium-hydrogen phosphate, tetra-butyl-ammonium hydroxide (as a 40% solution in water), acetonitrile HPLC grade, and concentrated HCl, were purchased from Fluka Chemie, Deisenhofen, Germany.

2.3. Chromatographic conditions

The nucleotides were separated on an Ultrasphere C_{18} column. The eluent was prepared with 0.1 *M* di-potassium-hydrogen phosphate and 1.0 m*M* tetra-butyl-ammonium hydroxide. The pH of the eluent was adjusted to 6.0 with concentrated HCl, and 3.5% (v/v) acetonitrile was then added. The eluent was sonicated to remove air bubbles. The flow rate was maintained at 1.4 ml/min. The separated nucleotides were detected at 254 nm. The output signal of 1 V from the detector was fed to the computer via the interface. The peaks of the nucleotides were integrated with the apex chromatography software, and external standards were used to identify and quantitate nucleotides in the unknown samples.

2.4. Standards

A number of 20-mg amounts of AMP, ADP, and ATP were each weighed in a 10-ml tube, dissolved in ice-cold water, aliquoted and deep-frozen; as necessary, these stock solutions were appropriately diluted with eluent. To study the elution positions of guanosine nucleotides (GTP, GDP, GMP) these materials were identically prepared.

2.5. Sample procurement

During the course of the present and previous [5] experiments it was found that samples must be maintained in a deep-frozen state, and that this is indispensable for preventing degradation of tissue ATP to ADP and AMP. Therefore, in the present experiments with male Sprague–Dawley rats (body weight ~280–300 g) the procedure was as follows: one full-length hind-leg of the anesthetized animal was shock-frozen in liquid nitrogen, within seconds amputated above the knee, and immediately stored at −80°C. Prior to analysis the leg was lyophilized and the tibia mechanically freed of all adherent soft tissue, divided into three portions of similar length (proximal, middle, distal), and finely powdered using a pestle and mortar. A group of intact animals was used as controls (Normal, $n=11$). In a second group, blood was withdrawn from the carotid artery until the systolic blood pressure measured 40 mmHg; the median periostal blood flow was ~22%, and the median blood oxygen partial pressure was ~31% of control values (for details see Ref. [6]); this situation was maintained over 30 min (Shock, $n=12$). In a third group blood withdrawal and shock induction were carried out in an identical manner, but the conserved (in citrate–glucose buffer) blood was retransfused within 15 min (Shock+Blood, $n=7$).

2.6. Measurement of unknowns

Methanol in a beaker and the sonicator were precooled by dipping into liquid nitrogen contained in a thermos flask. Powdered bone was weighed into a 2-ml Eppendorf vial, and kept in the precooled methanol; thereafter 300 μl HPLC eluent (see above) were added, immediately followed by sonication for 30 s, and the mixture vortexed for 15 s. After centrifugation at 4°C, 11 200 *g*, 30 s, the supernatant was injected into the HPLC system. Initially another 300 μl eluent were added to the pellet of the unknowns, and the whole vortexed and centrifuged for another measurement of residual nucleotides.

2.7. Calculations, presentation of data

Nucleotides were expressed as nmol per g dry bone. The adenosine nucleotide energy charge (EC) was calculated using Atkinson's formula [7]: ATP+0.5 ADP/(ATP+ADP+AMP); EC is dimensionless. For the three experimental groups the arithmetic mean (standard error) were given. Statistical comparisons were made using the *t*-test for unpaired observations, or the *U*-test, as appropriate.

3. Results

3.1. Specificity

The chromatogram obtained with the AMP, ADP and ATP standards, when loaded together with GMP, GDP and GTP standards, is shown in Fig. 1A. There was no overlap of the six peaks. GMP, GDP and

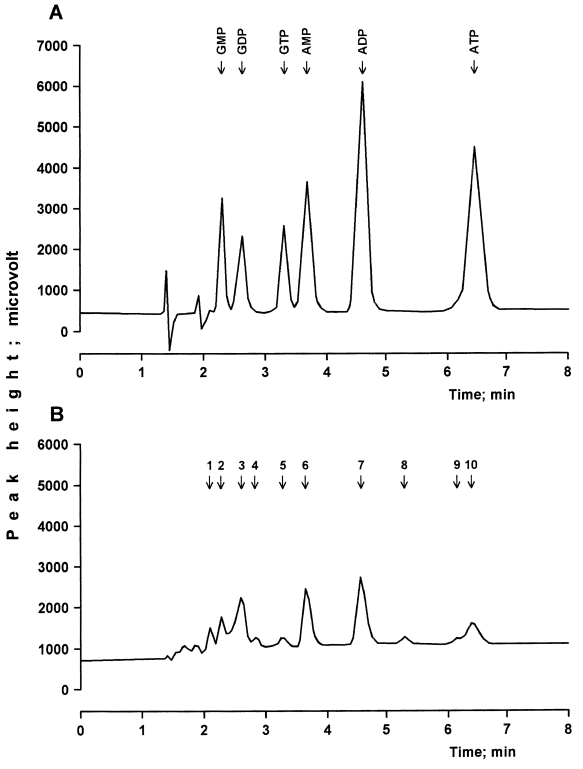

Fig. 1. HPLC of AMP, ADP and ATP. Conversion of *y*-scale into absorption units (AU) at 254 nm: 1 V=1 AU. (A) Elution positions of nucleotide standards. (B) Elution positions of nucleotides as contained in a pool sample of bone. The numbers and arrows denote peaks as follows: 1, unidentified; 2, GMP; 3, GDP; 4, unidentified; 5, GTP; 6, AMP; 7, ADP; 8 and 9, unidentified; 10, ATP. For other details see text.

GTP eluted at time points 2.3, 2.6 and 3.2 min, respectively, contrasting with the later elution positions of AMP (3.7 min), ADP (4.6 min), ATP (6.3 min). The adenosine nucleotides contained in a pooled sample from normal rat bone elute at the same position as the respective standard material, well separated from guanosine nucleotides, but several additional peaks remained unidentified (Fig. 1B). On addition of adenosine nucleotide standard material to another pooled sample only the respective peak increased (see also Analytical recovery, sample size below).

3.2. Extraction efficiency

On re-extraction of the pellet, ATP is too low to be measured, while a maximum of 10% AMP and 5% ADP, generally present in bone in much higher concentrations than ATP (Table 2), escapes extraction. The possibility of sub-optimal sample extraction, causing falsely low ATP, can therefore be ruled out, and may be negligible for AMP and ADP. It should be noted that complete separation of pellet and supernatant after the first extraction is virtually impossible, and may account for some nucleotide overspill to another extraction.

3.3. Sensitivity, linearity

AMP and ADP, eluting before ATP, elicit sharper peaks than the latter. The sensitivity of the method was adjusted to a signal-to-noise ratio of 3. Using the above conditions of sample preparation, the lowest measurable concentrations were 0.015, 0.02, and 0.04 nmol per 10-μl injection volume, for AMP, ADP, and ATP, respectively. When nucleotide standards in concentrations of up to three times the average nucleotide concentration of the normal proximal tibia were analyzed (see below), the measured values were found to be linear.

3.4. Analytical recovery, sample size

The data obtained on addition of ATP, ADP, and AMP to three individual samples of different weight from the proximal tibia are given in Table 1. The mean recovery (%) was 89.5, 99.9, and 96.8, for ATP, ADP, and AMP, respectively.

Table 1
Recovery of adenosine nucleotides added to dried, powdered and thereafter extracted bone

Sample	Nucleotide	Measured (nmol)	Added (nmol)	Recovery (nmol)	%
A	ATP	1.718	1.488	2.852	89.0
B	ATP	1.655	1.488	2.781	88.5
C	ATP	0.818	1.488	2.100	91.1
A	ADP	3.248	4.079	7.369	100.6
B	ADP	4.014	4.079	8.010	99.0
C	ADP	4.940	4.079	9.022	100.0
A	AMP	3.239	5.159	8.171	97.3
B	AMP	4.088	5.159	8.819	95.4
C	AMP	5.097	5.159	9.953	97.0

The weight of samples A, B and C was 10.1, 12.8 and 17.0 mg, respectively.

To establish whether the recovery of nucleotides, in particular ATP, depends on the size of the sample required for processing with 300 μl eluant, the sample weight was varied from 10 to 30 mg, and a weight-proportional linear increase in nucleotides (R^2=0.99; Fig. 2) was found. On this basis, and in order to minimize handling errors, samples weighing ~16–17 mg were used for routine work.

3.5. Repeatability, precision of duplicates

From each of the three anatomic tibial regions a larger pooled sample was prepared, which allowed the estimation of precision of repeat measurements

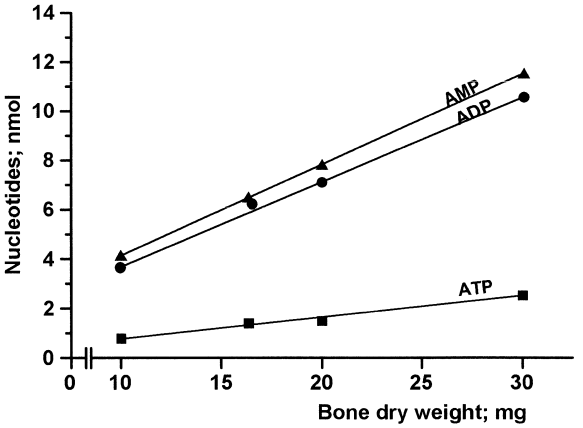

Fig. 2. Measurement of nucleotides in bone samples weighing from 10 to 30 mg. Note linearity.

(C.V. in series) and duplicates of individual samples. A total of ten replicates from the proximal, middle, and distal third yielded 350, 221 and 65 nmol/g ATP, 277, 224 and 82 nmol/g ADP, and 50, 27 and 15 nmol/g AMP, respectively; the corresponding C.V.s (% of standard deviation) were 2.6, 1.3 and 4.6 (ATP), 2.7, 1.6 and 4.6 (ADP), and 3.0, 2.2 and 4.5 (AMP).

The standard deviation (SD) of duplicate determinations of ATP, ADP, and AMP in 23 samples of proximal tibia was calculated using an established formula ($SD=\sqrt[2]{(\Sigma d^2)/2n}$, where d, Σ and n are the difference of two measurements of an individual sample, the sum of observed differences, and the number of duplicates, respectively). SD, expressed as a percentage of the mean, was 3 (ATP), 1 (ADP), and 1 (AMP). However, somewhat less precision was observed in a smaller series of the tibial middle and distal thirds (data not shown).

3.6. Nucleotides in bone (Table 2)

In all three anatomic regions of normal rat tibia, ADP and AMP are several times as high as ATP. Furthermore, there is an impressive downward shift in nucleotide content from proximal to distal. After induction of hemorrhagic shock, ATP and ADP in proximal tibia declined significantly, while mean AMP increased. Nucleotides returned almost to normal immediately following retransfusion at the end of the 30-min shock period. In the middle of the tibia — the area with the greatest portion of cortical versus cancellous bone, and a greater content of haemopoetically active bone marrow versus proximal and distal diaphyses — there was a shock-induced decline in all three nucleotides. Also in this region, ADP and AMP reached minimum levels despite the blood retransfusion. In the distal tibia, significant changes in nucleotides did not occur either after shock alone or after shock plus blood retransfusion. The mean total adenosine nucleotide content (ATP+ADP+AMP) was almost stable in proximal and distal tibia: 1059, 911, 939 and 128, 144, 145 nmol/g, for the groups Normal, Shock, and Shock+Blood, respectively. However, total nucleotide content varied markedly in middle tibia (Normal 506, Shock 282, Shock+Blood 169 nmol/g), indicating that adenosine nucleotide content of long bones not only depends on anatomical site, but also the conditions of investigation.

EC, by definition giving insight not only into ATP degradation but also the capacity of tissue to regenerate ATP from both AMP and ADP, declines from proximal to distal tibia. In all three groups EC was low, i.e. less than the value observed in parenchymatous organs [5] and mineralizing cartilage [8]. EC was significantly reduced by shock in all three tibial regions — least in the distal — but was completely normal in animals subjected to shock and blood retransfusion. Similarly, the mean ratio ATP/ADP — when $\leq$1.0 considered to indicate progressive mineralization, as shown for growth plate cartilage due to hypoxia in this region [8] — in normal rats was 0.21, 0.20 and 0.20, for proximal, middle and distal diaphysis, respectively; mean ATP/ADP in rats undergoing shock was 0.10, 0.10 and 0.14 (same order), but was 0.20, 0.18 and 0.22 (same order) in rats undergoing shock and blood retransfusion.

4. Discussion

The described HPLC methodology was previously used by us for the measurement of liver adenine nucleotides in the anatomically intact rat [5]; the ATP values then found were close to those obtained with the use of ^{31}P nuclear magnetic resonance spectroscopy (~12 (see Refs. [9–11]) vs. 11 (see Ref. [5]) μmol/g dry tissue). The latter method operates without the need of tissue extraction, hence is considered as reference. On this basis, the data obtained by us using HPLC for liver [5] and bone (present work) should have been unbiased by factors such as ATP loss owing to tissue ischemia during sample procurement, incomplete tissue extraction, inferior specificity, and other potential sources of error (for further comments on low ATP in bone see below).

Compared with current enzymatic methods, our HPLC analysis is not only highly sensitive (detection of nucleotides at the picomole level) but also robust inasmuch as interference by guanosine nucleotides and other substances could not be detected. For example, enzymatic analysis is plagued by contamination with ATP-hydrolyzing enzymes, and by 3-

glycerate kinase reaction with other than adenosine nucleotides, leading to falsely low ATP [13]. Luminometry, although easy to perform, quick and economic, is restricted to ATP [2,3,12], i.e. in general cannot provide insight into the dynamics of nucleotides involved in the numerous reactions that are characteristic of living cells and tissues, including bone. In contrast, with HPLC the three nucleotides of bone can be quantitated within a single run, together allowing total nucleotide content, ATP/ADP, and EC to be estimated. In our hands, measurement of ten samples per hour is feasible at a reasonable cost/efficiency ratio. Thus, once the HPLC equipment is available, the manpower of one experienced technician and the costs for one column (allowing analysis of ~300 unknowns) have to be calculated for a project such as that presented here.

ATP, involved in the regulation of fundamental cellular processes, in the metabolically highly active liver is two orders of magnitudes higher [5] than in proximal tibia (Table 2). A vast amount of literature on ATP is available not only for liver but also other vitally important tissues, such as the cardiovascular system, kidney, lungs and brain, contrasting sharply with bone. The reason for this discrepancy may be that to carry out in vitro studies in tissue and cells from the former sites is readily feasible, unlike in material from bone, which is more complex due to the organization into non-mineralized and mineralized regions. There is an urgent need for better understanding of in vivo metabolic events in bone, especially when associated with changes in microcirculation, oxygen supply, and tissue energy. This situation forced investigators to develop new strategies. The ''whole hindleg preparation'' of an experimental animal, as used here (see Materials and methods), may be a promising model: it shows for the first time that shock-inducible pronounced microcirculatory changes [6] are accompanied by changes of bone adenosine nucleotides, which are almost fully reversible upon appropriate treatment of shock (Table 2).

The low total nucleotide content of proximal tibia (contrasting with the eight times higher content reported for calcifying cartilage in long bone epiphyseal growth plate [8]), low EC (Table 2) and low ATP/ADP, are unexpected. However, there is circumstantial evidence that these findings reflect reality rather than some artifact related to ischemia during sample procurement. Although it was beyond the scope of the present work to discuss in more detail possible causes of the hitherto not reported variation of individual and total nucleotides in the three anatomic regions of the tibia, and why ATP/ADP and EC are lower than in non-calcified organs, a few remarks are made on the basis of available

Table 2
Adenosine nucleotides and the adenylate energy charge (EC) in three anatomic regions of rat tibia, measured under normal conditions (Normal), after 30 min of hemorrhagic shock (Shock), and after shock followed by normalization of circulating blood volume by retransfusion of blood (Shock+Blood)

	Nucleotides	Proximal	Middle	Distal
Normal,	ATP	106 (12)	44 (7)	11 (1)
$n=11$	ADP	515 (45)	224 (36)	57 (7)
	AMP	438 (33)	238 (30)	60 (4)
	EC	0.34 (0.01)	0.31 (0.01)	0.30 (0.01)
Shock,	ATP	32*** (4)	12*** (5)	8 (2)
$n=12$	ADP	307*** (23)	115* (36)	59 (10)
	AMP	572* (53)	155* (34)	77 (11)
	EC	0.21*** (0.01)	0.23*** (0.01)	0.26* (0.02)
Shock+Blood,	ATP	90 (15)	14* (2)	16 (4)
$n=7$	ADP	444 (48)	77* (14)	74 (17)
	AMP	405 (49)	78*** (12)	55 (8)
	EC	0.33 (0.01)	0.31 (0.02)	0.35 (0.02)

For details see Materials and methods section. Except EC (dimensionless), data are mean values (SE) of nmol/g dry tissue.
* $P \leq 0.05$ versus Normal.
*** $P \leq 0.001$ versus Normal.

knowledge. Firstly, in long bones of rats and humans there are anatomic peculiarities of the arterial vasculature: a principal nutrient artery in the proximal and leaving venous channels and the emissary veins in the middle and distal diaphysis [14,15], suggesting that oxygen supply also declines from proximal to distal. Secondly, a localized decrease in tissue oxygen tension shifts cellular aerobic to anaerobic metabolism [16], by other investigators considered as prerequisite for initiation of the complex series of intracellular events ultimately leading to extracellular deposition of mature crystals of hydroxyapatite, which in terms of tissue weight is the main constituent of cortical bone [17]. Thirdly, ATP in the extracellular fluid of mineralizing cartilage is in the nanomolar range, like in present work on bone, while ADP and AMP are somewhat higher; these findings render it doubtful that ATP itself is involved in extracellular mineral deposition [17]. Fourthly, also in calcifying epiphyseal rat cartilage, for which hypoxia has been demonstrated [16,17], ATP/ADP is ~1.0, but >1.0 in non-calcifying cartilage [16], corroborating the view that along the intra-bone mineralization process there is an enormous shift toward limitation of mitochondrial energy generation (for details see Refs. [3,17]).Taken together, the low ATP/ADP in tibia of present work (~0.2; Table 2) is interpreted to mean that it reflects the normal situation, i.e. unbiased by uncontrolled ATP degradation. Support for this view comes from the even lower ATP/ADP in rats undergoing shock, and the restoration of normal ATP/ADP in rats undergoing additional retransfusion of blood. Finally, the mentioned anatomical characteristics of the tibia, and the close coupling of ATP and EC under the described conditions, suggest that in long bones, but probably bones in general, there exists a feedback linking microcirculation, the metabolic activity of specialized bone cells such as osteoblasts and osteoclasts, and the levels of adenosine nucleotides. Indeed, ATP concentrations from 0.2 to 2.0 μmol in the bathing medium, considerably lower than ATP per g dry matter of bone in the present work, stimulate bone resorption-related morphological changes of osteoclasts as contained in cultures of bone cells of neonatal rats, while higher ATP inhibited osteoclast formation [18]. Monitoring of ATP from the fracture site of rabbit tibia revealed an early peak in callus at 2 weeks post-fracturing (~10 nmol/g dry matter), thereafter declining steadily to less than 1 nmol/g dry matter after 12 weeks, an order of magnitude seen in the intact contralateral tibia [3]. Such observations may advance our understanding of the role of energy nucleotides in bone resorption, formation, and remodeling cycles [18,19], and bone fracture healing [3], in addition the associated functional state of bone capillaries [20], the role played by vasoactive substances [21], and the resulting microcirculation.

In conclusion, adenosine nucleotides in long bones can be reliably measured by HPLC, provided the tissue has been obtained as described herein. Unlike the situation in non-calcified tissue, in the normal rat ATP in tibia is low, while ADP is high. Bone ATP and EC decline during shock, but normalize on appropriate treatment of shock.

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