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Sodium and potassium in the bones of penguin and skua revealed by EPR and SR-XRF technique

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Abstract Penguin and skua in the maritime Antarctic have high salt loadings in the body due to almost exclusive diet consumption of marine invertebrates. However, the storage and turnover of sodium and potassium in these animals are poorly investigated. Here we determined the concentration and microscopic distribution of the two elements in the bones of penguin and skua. The average concentrations of sodium and potassium in penguin bone were comparable with that in skua bone (0.18% and 0.82% for penguin bone; 0.19% and 0.76% for skua bone in dry weight). The ratios of sodium to calcium and potassium to calcium (0.0330 and 0.0075 for penguin, 0.0335 and 0.0082 for skua in average by weight) were somewhat higher than the reported ratios for terrestrial animals, indicating these marine animals' bone enrichment of salt. The ratios of sodium to potassium in average by weight were 6.75 and 4.65 for penguin and skua, respectively. This value is much lower compared with the bulk sea water ratio of about 27.0, implying that potassium is favorable to reside in the bone rather than sodium. Both sodium and potassium were found to significant correlation with the content of organic materials in bone based upon the intensity of native signal determined by electron paramagnetic resonance (EPR). It was estimated that almost all of potassium is kept within the organic phases, while about 30% of sodium is stored in organic phases and the other 70% within mineral phase. The microscopic distributions of potassium in the cross-section and/or surface were revealed by synchrotron radiation X-ray fluorescence (SR-XRF) technique. The ratio of potassium to calcium based upon the SR-XRF intensity counter varied considerably from the surface to the interior, and on the surface the highest concentration of potassium was observed in the middle section with decreasing amounts toward the edge. This indirectly documented that exchange of potassium between fluid and bone organic phase maybe occur.

Key words bone; penguin and skua; sodium and potassium; electron paramagnetic resonance (EPR); synchrotron radiation X-ray fluorescence (SR-XRF).

1 Introduction

Rankin *et al*. (2000) investigated the ionic composition in the snow samples taken at various distances from the emperor penguin (*Aptenodytes forsteri*) colony near Halley station in the maritime Antarctic and found extremely high potassium concentrations in and around the colonies[1]. The increased potassium was ascribed to the emissions from penguin faeces. This phenomenon subsequently rose the concerning on the turnover of salt and the other elements in penguin. For example, Xie and Sun (2003a) has reported fluoride content in bones of Adelie penguin (*Pygoscelis adeliae*) and environmental materials in Antarctica[2]. For the metabolism of salt, physiologists have long contended that consumption of a diet of marine invertebrates imposes a high salt load on animals. Since up to 90% of penguins' diet, especially Adelie penguins' diet, is marine invertebrates, usually krill (mostly *Euphausia superba*; Volkman *et al*. 1980), penguin has high salt load in the body[3]. Janes (1997) has reported the solute concentrations in salt-gland secretions, blood plasma, and urine of Adelie Penguin adults and chicks and the regurgitated food, and found that potassium relatively enriches in penguin body rather than sodium with a low Na:K ratio compared with marine signals[4]. However, up to date there are no reports on bone salt of penguin. It is known that approximately a third of the total body salt content of man, dogs, and rats is present in the inorganic portion of the skeleton[5]. The bone salt of penguin is thus urgent to be investigated.

The purpose of this study was to look at and get the baseline information of the storage of salt in penguin bone. The sodium and potassium concentrations were determined. In order to figure out whether salt reside in organic phase or mineral phase the electron paramagnetic resonance (EPR) technique was applied to analysis of the content of organic content in bone. Moreover, synchrotron radiation X-ray fluorescence (SR-XRF) approach was used to determine the microscopic distribution of potassium in the surface and cross section. As skua frequently preys penguin, the bones' salt of skua was also investigated in this note.

2 Materials and Methods

2.1 Sample Collection and Preparation

During the Fifteenth and the Eighteenth Chinese Antarctic Research Expeditions (December 1998-March 1999; December 2001-March 2002), fresh skeletons of Chinstrap penguin (*Pygoscelis antarctica*) and skua (*Catharacta maccormick*) on Ardley Island and fresh skeletons of Gentoo penguin (*Pygoscelis papua*), Adelie penguin (*Pygoscelis adeliae*) and skua (*Catharacta maccormick*) around the Zhongshan Station were collected, respectively[6].

A tool made of stainless steel and plastic was used to clean soft tissues and blood from the samples. Fat and marrow in the samples were removed by extraction with a mixture of chloroform and ethanol (v/v, 1:1) for 12 hours. The samples were then rinsed with deionized water in an ultrasonic cleaner several times, and air-dried in a

desiccator. Bone samples were firstly scanned by synchrotron radiation X-ray fluorescence (SR-XRF) to obtain the salt distribution in the surface and cross-section. Finally the samples were crushed in a mortar with a pestle and sieved to a grain size of 200 μm. Each sample was divided into several sub-samples for electron paramagnetic resonance (EPR) measuring and chemical composition analysis.

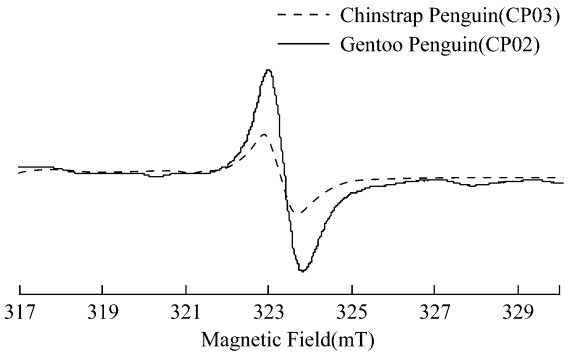

Fig. 1 The typical native EPR signal spectrum for the bones of penguins.

2.2 *Electron Paramagnetic Resonance (EPR) Spectra*

The EPR spectra were recorded on a JEOL JESFA200 spectrometer operating in the X-band at the Structure Research Laboratory of the University of Science and Technology of China. The measurements were performed using a standard cavity with a computer-interfaced spectrometer. The microwave power was kept constant at 1mW at room temperature, and the magnetic field modulation amplitude was 5G. Seven samples were measured under this condition. Two additional samples were stepwise heated in air from room temperature to 200℃. For in situ X-band measurements, a high temperature cavity was used. After each annealing period of 20 min at the selected temperature, the spectrum was measured at room temperature. The microwave and modulation were 1 mW and 3.5G, respectively. The MnO in MgO sample positioned in the cavity was used for an absolute g-value calibration. Each sample was composed of 400 mg powder.

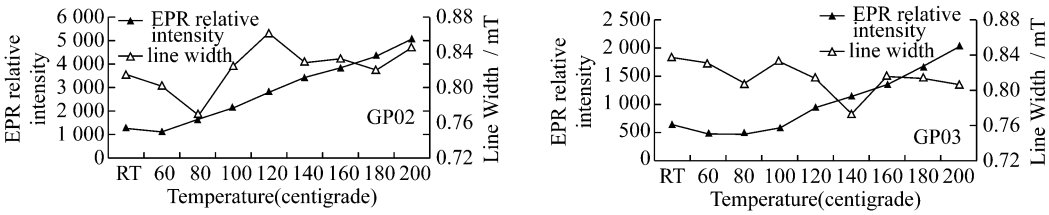

Fig. 2 Changes in the intensities of the native EPR signal in respond to the increase of heating temperature.

2.3 *Chemical Composition Analysis*

Powder bone samples were digested by multi-acid in a Pt crucible with electric

heating. The digested samples were analyzed for trace and major elements. Atomic absorption spectrophotometry (AAS) (model PE-1100, PerkinElmer, USA) was used to determine K, Na and Ca. Precision and accuracy of our results were monitored by analyzing standards with real samples in every batch of analysis. The analytical methods for these elements/oxides were reported in detail by Sun *et al*. (2000 and 2004) and Huang *et al*. (2009)[7-9].

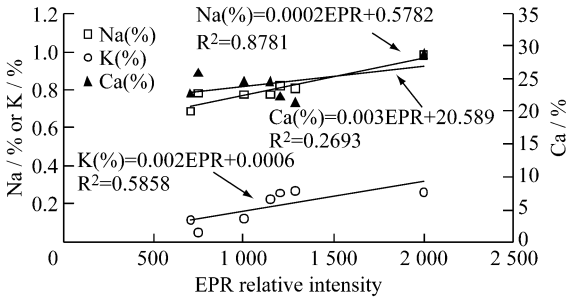

Fig. 3 Relationship for the concentration of Na and K in bone and the intensity of native EPR signal.

2.4 *Synchrontron Radiation X-ray Fluorescence (SR-XRF) Analysis*

The potassium distribution in the cross-section was calculated based upon our previous results reported by Xie *et al*. (2003b)[6]. The surface distribution of potassium was determined by SR-XRF at the synchrotron radiation microprobe XRF experiment station of Beijing Synchrotron Radiation Facility (BSRF) in March, 2004. The method was similar to the one described by Xie *et al*. (2003b)[6]. In brief, electron energy is 2.2 Gev, intensity is 40～80 mA, the energy of radiation is 4.0～30 keV, the exciting radiation is white light, and the beam line is 4W1B. Reflector is not used. The X-ray irradiation area touching the sample surface was working under liquid nitrogen and was placed 4cm away from the samples with energy resolution about 150～350 eVHWFM. The dead time rate of the detector was between 20～25%. A 2048 multichannel analyzer (MCA) was used to record and analyze the XRF spectrums. The sketch map of the experimental equipment was shown in our previous references[6,10].

Samples were immobilized on the sample platform about 1 meter away from the adjustable slits. The angles between the incident X-ray beam, the sample plane and the detector were 45 and 90, respectively. An optical microscope was used to adjust the position of the samples. The sites for 1-cm interval along a line from upside to downside of bone were analyzed. The effective time of X-ray irradiation was 100 seconds at room temperature. The qualitative experiment data was processed by software AXIL.

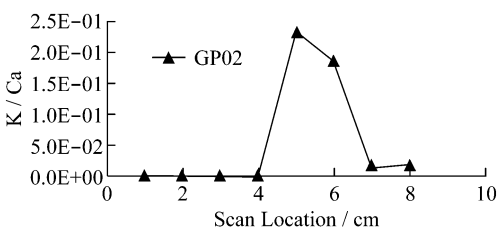

Fig. 4 The ratio of K/Ca along a line from the top to bottom of bone surface of penguin revealed by SR-XRF.

3 Results and Discussion

3.1 The contents of sodium and potassium and the ratios of Na/Ca, K/Ca and Na/K in bone

The contents of sodium and potassium by weight are shown in Table 1. Sodium ranges from 0.77% to 0.99% with 0.82 in average for penguin, which is comparable with the average value of 0.76 for skua. The average content of potassium for penguin is 0.18, almost equal to the that of skua as well. Potassium is about 4 times lower than sodium and shows considerable variation from 0.05 to 0.27 among species with coefficient variation (CV) at 47.3%, while sodium shows relatively stable with CV value of 11.3%. The ratios of Na/Ca and K/Ca by weight are 0.0332 and 0.0079, namely, 0.0289 and 0.0040 by Eq.. Both of Na/Ca and K/Ca ratios are about a quarter higher than the values for normal terrestrial animals (e.g., rats)[5], indicating these marine animals enrich sodium and potassium in bone.

Table 1. Native EPR signal intensities, g-values, and chemical compositions of the bones of Antarctic birds

Sample Number	Species	Bone Type	Na (%)	K (%)	Ca (%)	Na/Ca	K/Ca	Na/K	EPR Intensity	g-value
AP03	Adeline penguin (*Pygoscelis adeliae*)	Wing bone	0.78	0.22	24.5	0.0316	0.0091	3.48	1150	2.0051
GP01	Gentoo penguin (*Pygoscolis papua*)	Wing bone	0.99	0.26	29.0	0.0341	0.0091	3.75	2004	2.0056
GP02	Gentoo penguin (*Pygoscelis papua*)	Leg bone	0.81	0.27	21.3	0.0379	0.0126	3.00	1289	2.0053
GP05	Chinstrap penguin (*Pygoscelis antarctica*)	Wing bone	0.77	0.12	24.6	0.0313	0.0049	6.40	1006	2.0054
GP03	Chinstrap penguin (*Pygoscelis antarctica*)	Leg bone	0.78	0.05	25.9	0.0302	0.0018	17.14	745	2.0056
CSK03	Skua(*Catharacta maccormick*)	Wing bone	0.69	0.11	22.8	0.0302	0.0049	6.11	706	2.0053
ZSK05	Skua(*Catharacta maccormick*)	Wing bone	0.83	0.26	22.5	0.0367	0.0115	3.18	1206	2.0057
Penguin	average value		0.82	0.18	25.1	0.0330	0.0075	6.75		
Skua	average value		0.76	0.19	22.6	0.0335	0.0082	4.65		

The content of calcium is about 24.4% in average for penguin and skua, and comparable to the value of normal adults' human bone[11]. Assuming calcium content in penguin and skua fresh wet bone is equal to the that of normal adults of rats at 8000 mEq. per kilogram reported by Bergstrom *et al*. (1954)[5]. The contents of sodium and potassium in fresh wet bone are thus estimated at about 230 and 30 mEq. per kilogram. Sodium is about one and a half higher than the one in the penguin

blood plasma, while potassium is 6 times higher than the value in blood plasma reported by Janes(1997)[4]. The ratio of sodium to potassium ranges from 3.00 to 17.14 with the average value of 5.7, which is about 5 times higher than the ratio in the buck sea water. Obviously, potassium is more favorable than sodium to enrich in bone. It is thus inferred that the exchange process between extracellular and intracellular for potassium may be different from sodium. Further investigation is required to understand this mechanism.

3.2 The relationship for sodium, potassium and organic material detected by EPR

It is well known that approximately one third of the total body sodium content of man, dogs, and rats is present in the skeleton. Originally, K and Na are presumed to be mineral constituents, mainly located in the hydration layer of the bone crystals and as surface-bound[5,12]. It has been reported that some of the carbonate share a monovalent bond with calcium, and that the second carbonate valence was occupied by sodium, i.e. Ca-O-CO_2-Na. However, Bushinsky *et al*. (2000) recently argued that within bone the organic materials contains the majority of the sodium and potassium by looking at the different ratios of Na/Ca and K/Ca between original bone and bone without organic material removed by hydrazine (Hydr)[13]. As the total salt contents were found to be relatively high in penguin and skua's bone, we further investigated whether these monovalent ions reside within the mineral or organic phases of bone.

Commonly, the chemical reagent hydrazine (Hydr) is used to remove organic material of bone. However, this reagent is toxic. Here the physical technique EPR is thus applied to investigating the content of organic materials in bone, which is known to significant correlation with the intensity of the native EPR signal.

The X-band spectrum of penguin and skua exhibited a broad native signal around $g=2.005$ with a linewidth approximately 0.8 mT at room temperature, similar to the non-irradiated crushed human phalanxes[14]. However, the peaks around $g=2.007$ and 1.998 for steady CO_2^- component seemed to overlap. The typical examples of the EPR spectra observed were presented in Fig. 1. The bone samples showed very similar EPR spectra shape and structure without marked differences from sample to sample. However, the samples presented considerable variations of the EPR spectral intensity. The g-value and intensity for each sample are listed in Table 1. The origin of this signal is generally attributed to the organic matrix of hard tissues[15]. It has been proven that the EPR parameters of this native signal are independent of the absorbed dose[15]. Its intensity would increase when it is heated with temperature below 400°C[16].

Two penguin bone samples were thus stepwise heated in air from room temperature to 200°C. After each annealing period of 20min at the selected temperature, the spectrum was measured at room temperature. The intensity of the native EPR signal for all heat treatment temperatures (HTTs) is shown in Fig. 2. Below 80°C, the sig-

nal intensity was relatively constant. After that the signal intensity in both tissues linearly increased with the heat treatment temperature. In comparison with the Chinstrap penguin bone, the Gentool penguin bone had a higher rate increase (2.5 times) in intensity per centigrade increase in temperature, and the intensity reached about 5000 at 200°C. Since we did not have a suitable cavity for temperatures higher than 200°C, it is unclear at what temperature this increasing trend would cease. For HTTs below 200°C, the signal width was around 0.8mT with a small variation of coefficients (less than 5%). Our observation is in agreement with previous reports for tooth enamel and dentine[16], and implies the role of organic materials in the native EPR signal.

To examine the potential effect of bone organic in the variation of salt content in bone, the Pearson correlation among K, Na, Ca and the native EPR signal intensity were calculated and shown in Fig. 3. Calcium was not found to significantly correlate with the native signal, confirming that this signal is not mainly contributed from the minerals in bone. While strong correlations between K, Na and the native signal were observed. The intensity of EPR shows positive linear relationships with both Na and K in the bones. Assuming the signal value of EPR is zero for the linear equation for Na and K, respectively, it is inferred that almost all of potassium is kept within the organic phases, while about 30% of sodium was stored in organic phases and the other 70% within mineral phase.

3.3 The distribution of potassium in the surface and cross-section of bone revealed by SR-XRF

The contents of sodium in bone surface samples were under detection limit of SR-XRF and yield no data. Potassium was detected somewhere on the surface of GP02 sample with great CV of 102.9%, indicating the distribution was extremely heterogeneous. The highest potassium content was observed in the middle section of surface. The ratio of K/Ca along a line from the top to bottom is shown in Fig. 4, which displays decreasing amounts from middle section toward the edge. The reasons for these unhomogenous distributions are not clear. Bushinsky *et al*. (2000) reported that the organic material has fixed negative sites that normally are bonded with potassium[13]. Although potassium is less likely to participate in the exchange for calcium in the hydroxyapatite-type mineral lattice, it can exchange freely with the fluid surrounding bone. Exposure of bone to a lower systemic pH in vivo or acidic medium in vitro will lead to an egress of bone potassium in conjunction with an uptake of hydrogen ions. Giving that the pH values in extracellular fluid may change with respect to different species and the regulation mechanism of potassium subject to pH change in penguin bone is similar to the rats, potassium in the surface bone may be lost somewhere due to the disorder of acid-base balance in the body and then display unhomogeneous distribution.

The potassium over calcium ratio profiles in the cross-section of Adelie penguin bone was shown in Fig. 5. On the cross-section of fd-s1 and fd-s2, the ratio increases

from surface to medullary channel. On fp-s1 the highest value occur near the surface and the ratio decreasing toward the surface and interior. On fp-s2 the trend of ratio shows almost flat. Although the profile shows complex, the middle section of cross-section composed of cortical bone for all of the profile displays relative constant ratio of K/Ca with relative lower values, further indicating few potassium reside in mineral phase. Change in the ratio of K/Ca was found on or around exterior surface or interior medullary channel. This implies that exchange of potassium between fluid and bone organic phase may occur and the process may mainly occur outside or inside of bone.

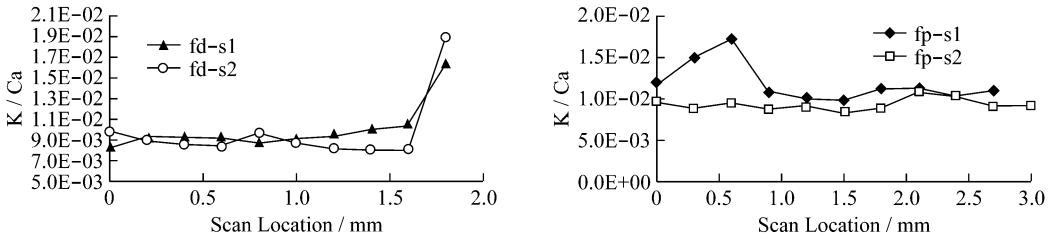

Fig. 5 The rations of K/Ca in the cross-section of penguin revealed by SR-XRF.

4 Conclusions

We have found that penguin and skuas' bone salt are relatively higher. In comparison with sodium, potassium is more favorable to reside in the bone. Almost all of potassium may keep within the organic phases of bone, while about 30% of sodium was stored in organic phases and the other 70% within mineral phase. This finding is in agreement with the distribution of sodium and potassium in mice bone investigated by high-resolution ion microprobe with secondary ion mass spectroscopy (SIMS)[13]. The microscopic distributions of potassium in the cross-section and/or surface determined by synchrotron radiation X-ray fluorescence (SR-XRF) were complex. Change in the ratio of K/Ca was found on or around exterior surface or interior medullary channel, implying that exchange of potassium between fluid and bone organic phase may mainly occur outside or inside of bone.

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