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Effects of Magnesium and Calcium on the Oxygenation Reaction of Erythrocruorin from the Marine Polychaete *Arenicola marina* and the Terrestrial Oligochaete *Lumbricus terrestris*

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ABSTRACT—Oxygenation function of annelid erythrocruorin (Er) is affected by Mg and Ca concentration in the blood. Four classes of responces may be encountered in different species: 1) Mg=Ca (equal effects), 2) Mg>Ca, 3) Mg<Ca and 4) no effect. In the marine polychaete *Arenicola marina*, at physiological pH and 20°C, Mg and Ca exerted almost equivalent effects in increasing oxygen affinity in the range of 1–200 mM. As measured from the slope of $\Delta\log P_{1/2} / \Delta\log$ [Cation] the effect of Mg was larger than that of Ca at the physiological concentration of respective ion (55 mM Mg; 10 mM Ca). The $n_{1/2}$ value was similar in the presence of both cations (pH 7.0) or higher for Mg (pH 7.6). In the terrestrial oligochaete *Lumbricus terrestris,* at the same condition, Ca was more effective than Mg, in raising oxygen affinity at both pHs, also at the physiological concentration (2–4 mM Mg; 8 mM Ca), and $n_{1/2}$ was similar for Mg and Ca (pH 7.0) or higher for Ca (pH 7.6). The Bohr factor, $-[\Delta\log P_{1/2} / \Delta pH]$, is maintained its maximum value within the span of the physiological concentration of Mg in *Arenicola*. In *Lumbricus*, Ca can contribute to the increase of the Bohr factor at the physiological concentration, but Mg cannot contribute to it. These results reveal that *Arenicola* and *Lumbricus* belong to classes 1) and 3), respectively, and that the oxygenation function of both Ers may be controlled by effective utilization of the more dominant of the divalent cations Mg and Ca.

Key words: annelids, erythrocruorin, oxygenation, Bohr effect, Mg/Ca effect

INTRODUCTION

In contrast to the vertebrate hemoglobin, where oxygen affinity is decreased by organic phosphates packaged in the red blood cells, the affinity of the high molecular-weight extracellular annelid respiratory proteins, termed either erythrocruorins (Ers) or hemoglobins, is increased by inorganic divalent cations. The effect of cations does not seem simply to be a charge effect since the effect of divalent cations is much greater than that of monovalent cations at double that concentration (Everaarts and Weber, 1974). Recent data suggest marked differences in the sensitivities of Er from different classes of annelids to Mg and Ca (Weber and Vinogradov, 2001). In marine polychaetes, the sensitivity shows much variation. In *Perinereis aibuhitensis*, Mg is more effective than Ca (Tsuneshige *et al*., 1989). In *Arenicola marina*, Mg and Ca exerts the same effect (Everaarts and Weber, 1974). In *Amphitrite ornata*, Ca is rather effective than Mg (Chiancone *et al*., 1981). In *Marphysa sanguinea*, Er appears to be insensitive to Mg and Ca (Imai *et al*., 1990), as is observed with *Eurythoe complanata* Er and Mg (Ilan *et al*., 1990). In oligochaetes, on the other hand, Ca is more effective than Mg in almost all species examined, as *Eisenia foetida* (Ochiai, 1984; Igarashi *et al*., 1985; Igarashi *et al*., 1991), *Lumbricus terrestris* (Fushitani *et al*., 1986), and *Pheretima hilgendorfi* (Ochiai *et al*., 1993). However, in *Glossoscolex paulistus*, Mg and Ca exert the same effect on the affinity at the physiological range (Marques and Meirelles, 1995). As an exception, the aquatic species *Tubifex hattai* shows no response to the divalent cations (Ochiai *et al*., 1991). In leech, Ilan and Harown (1993a,b) reported greater sensitivity to Ca than Mg in *Hirudo medicinalis* and *Macrobdella decora*, however, similar sensitivities to both cations at 50mM were observed in *M. decora* (Weber *et al*., 1995). The effect of divalent cations may be physiologically important in osmoconforming euryhaline animals like *Are-*

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nicola (Krogh-Rasmussen and Weber, 1979). Interaction with divalent cations is further of interest since it may change oxygen affinity of Er through alterations in proton concentrations at the molecular surface (Weber and Olsen, 1984).

The main aim of this study is to investigate differences in the sensitivities to Mg and Ca in annelids, and to discuss the relation between the sensitivity difference and ionic composition in the blood.

MATERIALS AND METHODS

Materials and preparations

The polychaete annelid, *Arenicola marina*, was obtained from Esbjerg on the west coast of Jutland, Denmark. Blood was collected as previously described (Krogh-Rasmussen and Weber, 1979). Blood was centrifuged at 14,000 rpm for 30 min to remove particulate matter, dialysed overnight against 100 fold excess of 0.01 M Tris-HCl buffer, pH 7.6 that was exchanged 4 times, and again centrifuged at 14,000 rpm for 30 min. The oligochaete annelid, *Lumbricus terrestris*, was collected in Aarhus and Frederikshavn, Denmark, and was identified according to the description of Sims and Gerard (1985). Blood was collected in glass hematocrit tubes as described for *Pheretima hilgendorfi* (Ochiai, 1983), centrifuged at 12,000 rpm for 5 min, pooled and diluted approximately 25 fold with 0.01 M Tris-HCl buffer, pH7.6, containing 0.5 mM PMSF as a proteolysis inhibitor (James, 1978). The Er was then precipitated by ultracentrifugation at 150,000 *g* for 1 hr (Beckman L8-70M, type 65 rotor) and gently resuspended in the same buffer without PMSF. After an additional centrifugation the pellet was suspended in a small amount of the buffer. Particulate matter was then removed by spinning at 21,800 *g* for 20 min. The Er samples were divided in 0.1 ml aliquots and were frozen at –80°C.

Oxygen equilibrium experiment

The aliquots were thawed individually on the same day as the oxygen equilibrium determinations were carried out. Before measurements, accurate volumes of standard cation solutions and 1 M BisTris propane or Tris buffers were added in the order and by the addition of distilled water to give differing cation levels, a final buffer concentration of 0.1 M and the same Er concentration in the individual tubes. All cations were added as chloride salts. Cation levels were assayed by measuring chloride concentrations using a Radiometer CMT 10 chloride titrator, and by atomic absorption spectroscopy. pH values were measured using a microelectrode set-up (Radiometer BMS 2 Mk 2 coupled to PHM64). Er concentrations were estimated from absorbances at 540 nm, using a millimolar absorption coefficient of 14.37 for human oxyhemoglobin (Assendelft, 1970), and methemoglobin contents were estimated by the spectral change of oxyhemoglobin at 574 nm. Oxygen equilibria were recorded with diffusion chamber modified after Sick and Gersonde (1969). Final heme concentrations were 1.4–1.5 mM in *Arenicola* Er and 0.8–1.1 mM in *Lumbricus* Er.

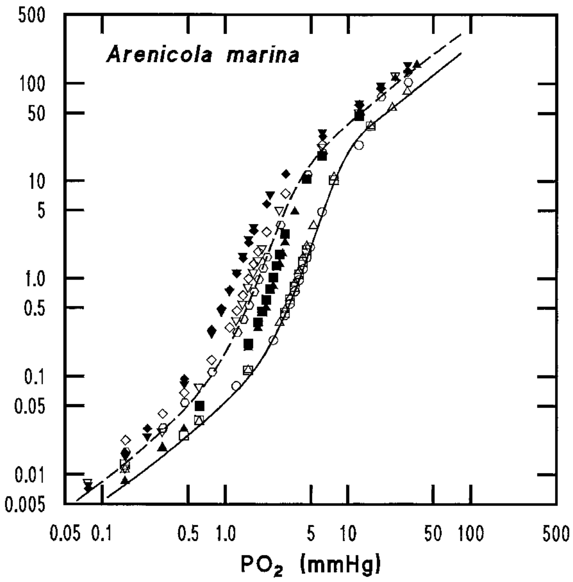

Fig. 1. Effects of Mg and Ca on the oxygen equilibrium curves of Er from *Arenicola marina*. Buffer: 0.1 M BisTris propane at pH 7.0 (6.98–7.01) and 0.1 M Tris at pH 7.6 (7.58–7.70), 20°C. pH 7.0: —○—, no additive (1.88 mM Mg, 1.03 mM Ca); △, 8.20 mM Mg; □, 11.7 mM Ca; ▲, 149 mM Mg; ■, 166 mM Ca. pH 7.6: - -○- -, no additive (1.60 mM Mg, 1.06 mM Ca); ▽, 9.30 mM Mg; ◇, 12.9 mM Ca; ▼, 157 mM Mg; ◆, 129 mM Ca.

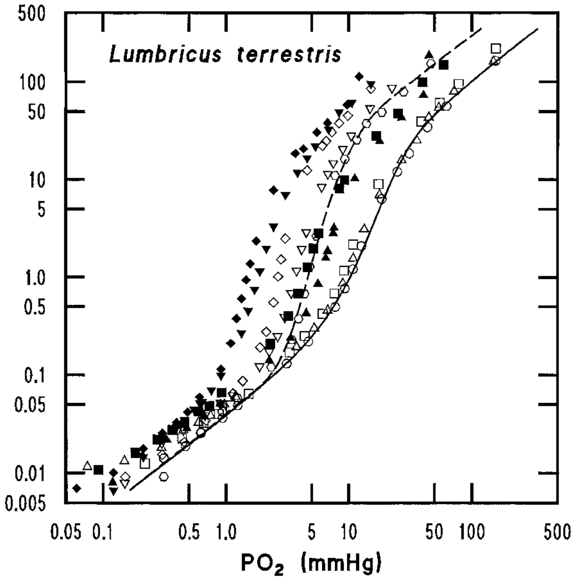

Fig. 2. Effects of Mg and Ca on the oxygen equilibrium curves of Er from *Lumbricus terrestris*. Buffer: 0.1 M BisTris propane at pH 7.0 (6.99–7.04) and 0.1 M Tris at pH 7.8 (7.72–7.80), 20°C. pH 7.0: —○—, no additive (0.20 mM Mg, 1.70 mM Ca); △, 9.60 mM Mg; □, 12.4 mM Ca; ▲, 160 mM Mg; ■, 170 mM Ca. pH 7.8: - -○- -, no additive (0.20 mM Mg, 1.55 mM Ca); ▽, 9.50 mM Mg; ◇, 11.8 mM Ca; ▼, 170 mM Mg; ◆, 162 mM Ca.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the effects of Mg and Ca on the oxygen equilibrium curves of Er from *Arenicola marina* at pH 7.0 and 7.6 and of Er from *Lumbricus terrestris* at pH 7.0 and 7.8 on the same scale. At pH 7.0, Mg and Ca levels of 10 mM exerted no effect on the oxygenation curve of *Arenicola* Er (Fig. 1), whereas 150–170 mM Mg and Ca increased oxygen affinity. At pH 7.6, 9–13 mM Mg and Ca slightly increased oxygen affinity, whereas more marked increases were induced by 130–160 mM Mg and Ca. At both pH values, no significant difference was found between the effects of these two cations. In *Lumbricus* (Fig. 2), 10–12 mM Mg and Ca did not give a remarkable effect at pH 7.0, but definite increase of oxygen affinity was observed at pH 7.8. The effect of Ca was obviously larger than that of Mg especially at pH 7.8. At both pHs, 160–170 mM Mg and Ca induced marked increase in oxygen affinity, the effect of Ca was somewhat larger than that of Mg. When compared with the oxygenation curve in the absence of the cations and that in the presence of the cations, both curves overlapped with each other at low oxygen saturation, but did not overlap at high saturation, and this phenomenon was more evident in *Lumbricus* than in *Arenicola*. This result implies preferential binding of both cations to oxygenated high affinity R (relaxed) state form of *Arenicola* and *Lumbricus* Ers, and the existence of at least two high affinity R states (Weber, 1981; Weber and Vinogradov, 2001). However, a resonance Raman spectroscopic study of *Lumbricus* deoxygenated low affinity T (tense) state and oxygenated R state showed no spectral changes involving the iron-proximal histidine stretching mode (Vidugiris *et al.*, 1993). Moreover, small angle X-ray scattering experiments of *Lumbricus* Er revealed that the overall volume almost unaltered upon deoxygenation (Krebs *et al.*, 1996).

Figs. 3 and 4 illustrate the concentration dependence of the effects of the cations on $P_{1/2}$ (oxygen tention at half-saturation) and $n_{1/2}$ (cooperativity coefficient at half-saturation) at pH 7.0 and 7.6/7.8 in *Arenicola* and *Lumbricus*, respectively. As evident from Fig. 3a, Mg and Ca exerted identical effects in increasing oxygen affinity of *Arenicola* Er at both pH values. At pH 7.0, increasing concentrations of both Mg and Ca raised $n_{1/2}$ values, from about 3.5 to 4.5. At pH 7.6, $n_{1/2}$ was almost unaffected at about 3.5 by Mg at all concentrations tested, and by Ca at concentrations below approximately 30 mM. At higher Ca levels $n_{1/2}$ fell to 2.5-3.0 (Fig. 3b). In *Lumbricus*, Ca was more effective than Mg in raising oxygen affinity at both pH 7.0 and 7.6, and the difference between the Mg and Ca effects were somewhat larger at pH 7.6 than pH 7.0 (Fig. 4a). As regards $n_{1/2}$, at pH 7.8 the value was almost constant at about 6–7 at Mg and Ca concentrations up to 50 mM, then gradually decreased to reach 3–4 at concentrations near 500 mM. Near 10 mM, however, Ca gave somewhat higher $n_{1/2}$ value than Mg. At pH 7.0, the value was 2.8 up to 10 mM Ca and Mg and then increased to 3.2–4.1 at higher cation levels (Fig. 4b). $n_{1/2}$ at pH 7.8 were much higher than those at pH 7.0, especially at low concentration of the cations, which is reverse compared to *Arenicola*. These results show $P_{1/2}$ of *Arenicola* Er has almost the same reactivity to Ca and Mg, whereas *Lumbricus* Er shows higher reactivity to Ca than to Mg on both pH values tested. Moreover, $n_{1/2}$ of *Arenicola* Er shows the same or higher reactivity to Mg, whereas *Lumbricus* Er shows the same or higher reactivity to Ca between the two cations. Another illustration of the cation concentration dependency of $P_{1/2}$ is furnished by a comparison of $\Delta\log P_{1/2} / \Delta\log$ [Cation] at 1–200 mM cation level represented by Table 1. It is evident that *Lumbricus* Er is 1.2 and 1.6 fold sensitive than *Arenicola* Er for Mg and Ca at pH 7.0, respectively, and 1.7 and 2.0 fold sensitive for each cation at pH 7.6, respectively.

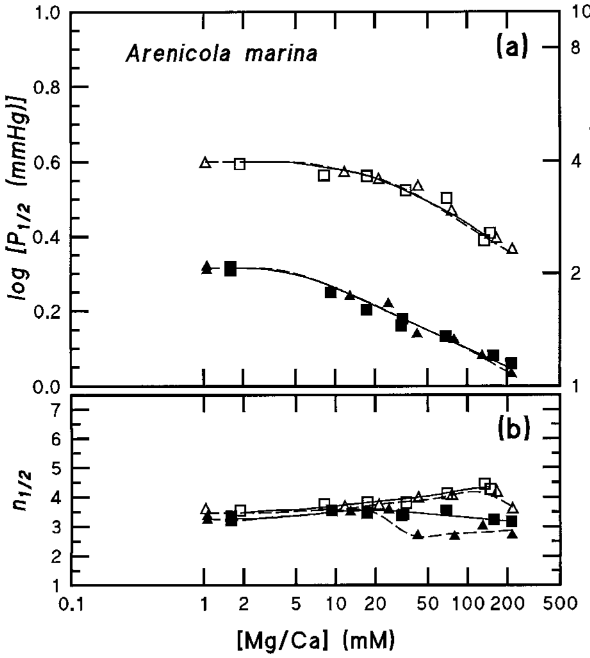

Fig. 3. Mg and Ca concentration dependency of log $P_{1/2}$ (a) and $n_{1/2}$ (b) of Er from *Arenicola marina*. 0.1 M BisTris propane buffer at pH 7.0 (open symbols) and 0.1 M Tris buffer at pH 7.6 (closed symbols), 20°C. (—□—, —■—) Mg and (- -△- -, - -▲- -) Ca.

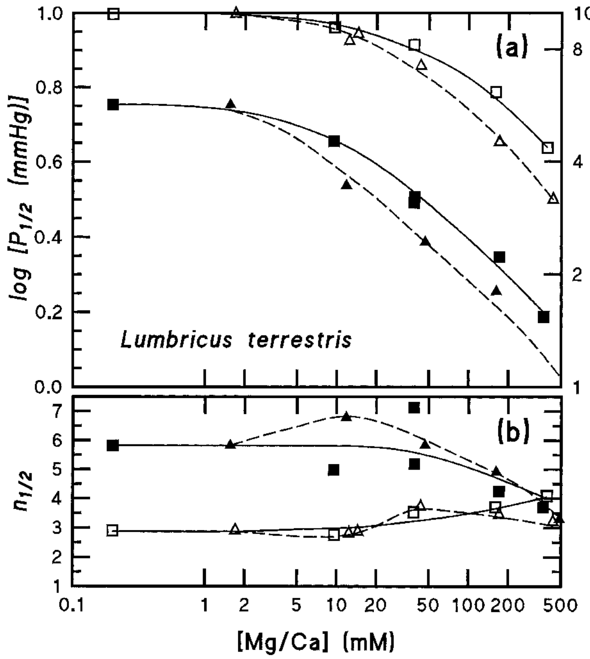

Fig. 4. Mg and Ca concentration dependency of log $P_{1/2}$ (a) and $n_{1/2}$ (b) of Er from *Lumbricus terrestris*. 0.1 M BisTris propane buffer at pH 7.0 (open symbols) and 0.1 M Tris buffer at pH 7.6/7.8 (closed symbols), 20°C. The values of $P_{1/2}$ at pH 7.8 were corrected to those at pH 7.6. $n_{1/2}$ values are those at pH 7.8. (—□—, —■—) Mg and (- -△- -, - -▲- -) Ca.

According to a bracelet model for *Lumbricus* Er proposed by Vinogradov and his collaborators, the hexagonal

Table 1. Cation concentration dependency of $P_{1/2}$ between 1 and 200 mM cation ($\Delta\log P_{1/2}/\Delta\log$ [Cation]) in the Ers from *Arenicola marina* and *Lumbricus trrestris*

	$\Delta\log P_{1/2}/\Delta\log$ [Mg]		$\Delta\log P_{1/2}/\Delta\log$ [Ca]	
	pH 7.0	pH 7.6	pH 7.0	pH 7.6
Arenicola marina	0.100	0.117	0.100	0.126
Lumbricus terrestris	0.117	0.204	0.161	0.252

bilayer structure is retained 12 dodecamers, consisting of three copies each of disulfide bonded trimer and monomer chains (12 × [a + b + c]3 [d3]), which are decorated with 36–42 linker chains. Chain a and one of the linkers are glycosylated (Vinogradov *et al.*, 1986; Vinogradov *et al.*, 1991; Martin *et al.*, 1996). *Arenicola* Er shows indistinguishable whole feature from that of *Lumbricus* Er, however, its proposed dodecamer composition is one trimer with nine monomer chains, six groups of the dodecamer are assembled and the center of which contains two extra copies of trimers, and its two sets are decorated with 42 linker chains. *Arenicola* Er does not have glycosylated chains in contrast to *Lumbricus* Er (Zal *et al.*, 1997). On the basis of a molecular model for *Lumbricus* Er proposed by Taveau *et al.* (1999), Kuchumov *et al.* (2000) classified the Ca^{2+}-binding sites of the Er into three groups by a relative affinity when compared to Ca and EDTA. According to the model, the low-affinity type (i) sites of the three, more than 100 sites in number with affinities lower than EDTA, involve the effects of 1–100 mM Group IIA cations on the Er structure and function. Most of the type (i) sites distribute to one of the linker chains (L3) and partly occur within the dodecamer subassembly. Because of the type (i) sites predominate in the linker chain, it is possible to attribute the difference of the Mg/Ca reactivity between both Ers to that of the linker chain content or the participation of glycosilation. However, the content is very similar in both Ers (Martin *et al.*, 1996; Zal *et al.*, 1997), and the L3 chain of *Lumbricus* Er is not glycosylated (Martin *et al.*, 1996).

Unfortunately, the available information on the Ca^{2+}-and/or Mg^{2+}-binding site of *Arenicola* Er is limited. Weber and Olsen (1984) observed that the Mg titration of *Arenicola* Er induced proton release from its molecular surface. In this experiment, a similar decrease in pH was found when Ca was added to the Er solution as well. These facts are an indirect but a clear evidence indicating the presence of Mg^{2+}-binding sites and another Ca^{2+}-ones for *Arenicola* Er. In this Er preparation, *Arenicola* Er retains approximately 170 Mg and 100 Ca per mole, and *Lumbricus* Er contains 27 Mg and 220 Ca per mole (see legends of Fig. 1 and Fig. 2). Especially in *Lumbricus* Er, each value is somewhat higher than that of the Ca reported by Standley *et al.* (1988) and those of the Mg and Ca for *Pheretima hilgendorfi* (Ochiai *et al.*, 1993), respectively. This may simply be due to the fact that dialysis step was omitted in this preparation, however, their numerical values may indicate the specific affinity of Ca for the Er. On the other hand, *Arenicola* Er, which includes dialysis in the process of preparation, still retains comparable amount of Mg in addition to Ca. This fact suggests the presence of at least two kinds of binding sites, one for Mg^{2+} and the other for Ca^{2+}, for *Arenicola* Er, and that these sites constitute the type (ii), type (iii), and a part of type (i) sites by analogy with *Lumbricus* Er (Kuchumov *et al.*, 2000). Anyway, classification of the binding sites and their identification for *Arenicola* Er must wait for future research.

Very recently, Jouan *et al.* (2001) classified annelid Ers possessing a hexagonal bilayer structure into two groups by cryoelectron microscopic observation. Type-I appears as the vertices of the upper layer are 16° clockwise rotated with respect to those of the lower layer. Type-II has two eclipsed hexagonal layer structure. Type-I occurs in oligochaete like *Lumbricus*, achaete and vestimentifera. Type-II is found in polychaete, the representative is *Arenicola*. As linker mediated equatorial or axual interactions are believed to contribute the high cooperativity of annelid Ers (Lamy *et al.*, 1996), the possibility cannot be excluded that the presence or absence of the 16° rotation participates in the function of both Ers.

Fig. 5 shows the Bohr factors ($-[\Delta\log P_{1/2}/\Delta pH]$) at various concentrations of Mg and Ca observed in the pH 7.0–7.6 in *Arenicola* and pH 7.0–7.8 in *Lumbricus*. In *Arenicola*, the value is highest (near –0.6) at 30–80 mM Mg and Ca concentrations, but decreases both low and high levels of the cation concentrations. In *Lumbricus*, the value begins to increase from –0.4 at 5 mM Mg and Ca, then reaches the maximum value of –0.75 at 40 mM Mg and Ca, and maintains the same value above 40 mM Mg and Ca. Ca concentration in the blood is about 10 mM in both *Arenicola* and *Lumbricus*, while Mg concentration is far higher in *Arenicola* than in *Lumbricus* (55 mM and 2–4 mM, respectively: Kamemoto *et al.*, 1962; De Jorge *et al.*, 1965; De Jorge and

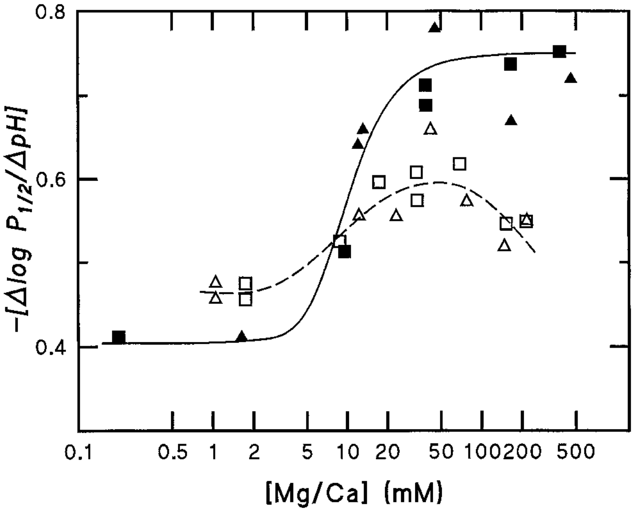

Fig. 5. Effects of Mg and Ca on the Bohr effects of Ers from *Arenicola marina* and *Lumbricus terrestris.* Bohr factors were calculated from the log P1/2 values at pH 7.0 and 7.6 in *Arenicola* (open symbols, broken line), and at pH 7.0 and 7.8 in *Lumbricus* (closed symbols, solid line). (□, ■) Mg and (△, ▲) Ca.

Table 2. Effects of Mg and Ca on the oxygen affinity of annelid Er

Polychaeta		
Mg Ca	*Arenicola marina*	Everaarts and Weber (1974); Present study
Mg > Ca	*Perinereis aibuhitensis*	Tsuneshige *et al.* (1989)
Mg < Ca	*Amphitrite ornata*	Chiancone *et al.* (1981)
no effect	*Marphysa sanguinea*	Imai *et al.* (1990)
Oligochaeta		
Mg Ca	*Glossoscolex paulistus*	Marques and Meirelles (1995)
Mg < Ca	*Eisenia foetida*	Ochiai (1984); Igarashi *et al.* (1985, 1991)
〃	*Lumbricus terrestris*	Fushitani *et al.* (1986); Present study
〃	*Pheretima hilgendorfi*	Ochiai *et al.* (1993)
no effect	*Tubifex hattai*	Ochiai *et al.* (1991)
Hirudenea		
Mg < Ca	*Hirudo medicinalis*	Ilan and Haroun (1993a)
〃	*Macrobdella decora*	Ilan and Haroun (1993b)

Sawaya, 1967; Prosser, 1973; Oglesby, 1978; Ochiai *et al.*, 1993). Here again we try to compare the reactivity of both Ers to each cation under their physiological concentrations. In *Arenicola*, the reactivity as measured from the slope of the tangential line of log $P_{1/2}$ vs log [cation] curve at the physiological concentration of respective ion (55 mM Mg; 10 mM Ca) is larger for Mg than that for Ca, especially at pH 7.0 (Fig. 3a). In *Lumbricus*, the slope for Ca is larger than that for Mg at pH 7.6 but similar at pH 7.0, at physiological concentrations of respective ion (2–4 mM Mg; 8 mM Ca) (Fig. 4a). These results reveal that the oxygen affinities of both Ers may be controlled by effective utilization of the more dominant of the divalent cations Mg and Ca. The Bohr factor may also be under the control of each cation concentration. In *Arenicola*, the blood concentrations of Mg and Ca decrease sharply upon transfer from sea water to brackish water from about 60 to 20 mM and 8 to 3 mM, respectively (Krogh-Rasmussen and Weber, 1979). However, as evident from Fig. 5, the Bohr factor is maintained its maximum value within the span of the physiological concentration of Mg, so it is considered Mg is made efficient use through the rise of the Bohr factor for the oxygen delivery between blood and tissues. In *Lumbricus*, as shown in Fig. 5, the effects of Ca and Mg on the rise in Bohr factor is much larger than those of the case of *Arenicola* at more than 10 mM. However, at physiological concentration of respective ion, Mg cannot contribute to the increase of the Bohr factor, but Ca can contribute to it. For this terrestrial earthworm, the increase of carbon dioxide concentration may be more important than the change of ionic concentration in the milieu on the Bohr effect (Weber and Baldwin, 1985).

The effect of Mg and Ca on the oxygen affinity of annelid Er is summarized in Table 2. Four cases of responses may be encountered in different species: 1) Mg=Ca (equal effects), 2) Mg>Ca, 3) Mg<Ca and 4) no effect. This report revealed that *Arenicola* and *Lumbricus* belonged to classes 1) and 3), respectively. In contrast to marine polychaetes that are represented in all four classes 1) – 4), terrestrial oligochaetes fall into the classes 1) and 3). This difference may relate to the specific habitats of the two animal groups.

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