LIGAND ACTIVITY IN THE CLEARANCE OF METALS
FROM THE BLOOD OF THE CRAYFISH
(AUSTROPOTAMOBiUS PALLiPES)

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SUMMARY

1. The rate at which metal ions are cleared from the blood of the crayfish
   Austropotamobius pallipes can be described as the sum of fast and slow
   exponential processes.

2. The rate constants of these processes were determined and discussed
   in terms of ligand chemistry.

3. The half life ($t_{1/2}$) of the fast component shows a typical Irving-
   Williams series for the various metals. This is interpreted as showing that
   the retention of metals in the blood is due to binding to protein ligands.

4. The concentrations of metals in the various tissues also reflect their
   binding characteristics. Class (a) metals (Ca and Sr) become associated with
   oxygen donors in the exoskeleton and stomach. Class (b) and borderline
   metals become bound to nitrogen and sulphur donor ligands in the
   hepatopancreas. The rapid disappearance of Cd$^{2+}$ and Zn$^{2+}$ from the blood
   can be attributed to specific ligands in the hepatopancreas.

INTRODUCTION

The role of metal ions in cell physiology has been considered mainly in relation to
the bulk electrolytes (Na, K, Ca, Mg) and their involvement in electrical and osmotic
phenomena. The trace metals (Mn, Fe, Co, Cu, Zn, Mo, V, Cr, Sn, Ni and Al) are
all essential for life but their biological importance has been studied mainly by
nutritionists and enzyme chemists. Few physiological principles have been
established (see Harrison & Hoare, 1980), except in studies on ion transfer across
membranes (Williams, 1981) and the relative affinities of ligands for acceptor ions
(Ahrland, Chatt & Davies, 1958).

Plasma membranes are hydrophobic structures and inorganic ions are hydrophilic
particles. As a result, membrane-enclosed spaces may be considered as compartments
in which large concentrations of charged particles may be accumulated and used as
sources of energy as long as their movements are controlled by suitable ligands. Thus
systems such as the control of the transfer of metals from one ligand to another in
metalloprotein syntheses are envisaged as a cascade effect involving their binding to
molecules of ever increasing strength and specificity (Williams, 1981). Ligands may,

Key words: Metal metabolism, ligand binding, crayfish.
therefore, not only provide a means for transporting ions across membranes and for incorporating them at particular sites but they will also separate them according to their chemical reactivities.

The general rules that govern the reactivities of metals to ligands depend upon their relative ionic and covalent bonding properties. On the basis of this a metal can be considered as class \( (a) \) (hard acid), class \( (b) \) (soft acid) or borderline (Arhland et al. 1958). Class \( (a) \) metals such as \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) have a ligand preference in the order \( \text{O} > \text{N} > \text{S} \) which in biological systems means that they attach to carbonate, sulphate, phosphate, carboxylate, carbonyl and alcohol groups. Class \( (b) \) metals such as \( \text{Cu}^+ \) and \( \text{Hg}^{2+} \) have the opposite order of preference i.e. \( \text{S} > \text{N} > \text{O} \) and consequently bind strongly to sulphhydryl, amino and imidazole groups. Many of the metal ions of interest in biological systems are technically ‘borderline’ in their properties e.g. \( \text{Fe}^{2+} \), \( \text{Co}^{2+} \), \( \text{Ni}^{2+} \) and \( \text{Zn}^{2+} \) but they are often associated with nitrogen or sulphur ligands.

There are several reasons why trace metals have tended to be ignored in cell physiology. One of these is undoubtedly the basic technical problem of measuring quantities which, in terms of reactive ion concentrations, are probably in the order of \( 10^{-10} \text{ mol dm}^{-3} \). Perhaps even more important, however, is the difficulty of interpreting the results in some meaningful way that opens up new experimental approaches. We have approached these problems in the simplest way possible by assuming that inorganic physiology is directly governed by the reactivity of metal ions with ligands. We have therefore measured the rates at which metals are removed from the blood of the crayfish and accumulated in the tissues. We have then attempted to see if the results obtained can be explained by the direct application of the rules governing the binding of metals to ligands. It should be emphasized, therefore, that the aim of this study is to see if the principles of metal-ligand binding can be applied directly to the physiological processes involved in the accumulation of metals in tissues.

**MATERIALS AND METHODS**

The crayfish (*Austropotamobius pallipes*) was used throughout this work since it is a well studied animal which has a large blood volume that can be sampled repeatedly. The animals were collected (by diving) at a reservoir near Banbury, Oxfordshire and comprised a single and apparently homogeneous population. They were maintained in aerated tap water in large plastic tanks, and fed on commercial fish pellets (Pond Pride, BP Nutrition). Intermoult male animals were acclimated to 5°C and starved for 24 h before use so as to minimize the effects of sex, temperature and the digestive cycle.

Crayfish were injected under the posterior edge of the cephalothorax with weighed aliquots of a variety of salt solutions. These consisted of \( ^{45}\text{Ca} \), \( ^{85}\text{Sr} \), \( ^{54}\text{Mn} \), \( ^{59}\text{Fe} \), \( ^{60}\text{Co} \), \( ^{64}\text{Cu} \), \( ^{65}\text{Zn} \) and \( ^{109}\text{Cd} \) injected as chlorides except for iron which was given as a citrate. The radioisotopes were obtained from Amersham International except for \( ^{64}\text{Cu} \) which was prepared by neutron activation of CuO at the London University Reactor Centre, Silwood Park. Each injection solution was made to contain approximately 0·75 \( \mu \text{mol} \) metal by addition of stable salts.

Blood samples were taken from the haemocoel using the opposite side of the cephalothorax to avoid possible contamination from the injection site. Aliquots o
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0.05 cm$^3$ were taken at times from 1 to 48 h after injection, when the animals were killed, dissected and tissues taken for further analysis. Tissue samples were analysed for γ-emitting radioisotopes using an Ultragamma 1280 (LKB-Wallac) while β emitters were measured in a TriCarb 2425 liquid scintillation counter (Packard Instruments). Where appropriate, corrections were made for radioactive decay during the experiment ($^{64}$Cu) and for quenching ($^{45}$Ca). Analyses of total metal were made by atomic absorption spectroscopy (Varian 175). At least four animals were used for each set of experimental values.

RESULTS

To calculate the rate at which metals are lost from the haemocoel it is necessary to know the volume of the haemolymph. The volume of distribution ($V_{\text{dist}}$) of the injected metal can be calculated from the equation

$$V_{\text{dist}} = \frac{\text{d.p.m. (inj)}}{\text{d.p.m. (time 0)}} \times \text{sample vol (cm}^3\text{)},$$

where d.p.m. (inj) is total counts injected and d.p.m. (time 0) is counts per sample volume at time zero.

The d.p.m. (time 0) value was obtained by extrapolating the rate of loss of radioactivity from the blood back to time zero and the volume of distribution ($V_{\text{dist}}$) was assumed to be equal to the blood volume. Results obtained in this way were in keeping with the empirical finding that blood volume was equal to 25% body mass (Gladwell, 1973). Using these methods the percentage loss of metals from the haemolymph was determined (Table 1). The results show that metals are lost from the blood in an exponential way but the initial rate of loss and the long term rate of loss vary for each metal (Fig. 1). It was therefore assumed that there are at least two types of process involved in this effect, namely, those predominantly due to ligand binding and those caused by other factors (e.g. excretion). If this assumption is correct the 'blood loss' curves can be resolved as the sum of two exponentials

$$C_m = Ae^{-k_1t} + Be^{-k_2t},$$

where $C_m$ is the percentage of metal in the haemolymph, $k_1$ and $k_2$ are rate constants for the fast and slow components respectively, A and B are constants and $t$ is time.

The value of this analysis is that it simplifies the quantification of the initial rate of loss of isotopes which, as can be seen from the examples in Fig. 1, is otherwise difficult. By using semi-logarithmic plots and the technique of curve stripping (Riggs, 1963), it was found that each process is a first order activity with regard to metal concentration, and rate constants were determined. These are shown in Table 1 together with an estimate of the statistical significance of $k_1$, the fast process that is attributed to ligand binding. Since the rate constant is related to the half-life by the expression $t_{1/2} = 0.693/k$ it is also possible to calculate the half-lives for the fast and slow processes involved in removing each metal from the blood (Table 2).

Analyses of the tissues collected 48 h after injection showed that the class (b) metals were accumulated in a typical sequence i.e. hepatopancreas >> antennal gland > gills > stomach, carapace and muscle, whereas the series was reversed for the class (a)
Table 1. Percentage of the dose of each metal remaining in the blood at various times after injection

<table>
<thead>
<tr>
<th>Metal</th>
<th>0.5 h</th>
<th>1.0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>24 h</th>
<th>48 h</th>
<th>k₂</th>
<th>k₁</th>
<th>r (k₁)</th>
</tr>
</thead>
<tbody>
<tr>
<td>⁶⁶⁴Cu</td>
<td>—</td>
<td>94 ± 7</td>
<td>84 ± 6</td>
<td>70 ± 4</td>
<td>56 ± 3</td>
<td>13 ± 3</td>
<td>5 ± 2</td>
<td>0.0266</td>
<td>0.00734</td>
<td>0.999***</td>
</tr>
<tr>
<td>⁶⁶⁶⁴Zn</td>
<td>114 ± 6.5</td>
<td>74 ± 20</td>
<td>—</td>
<td>70 ± 7</td>
<td>42 ± 7</td>
<td>7 ± 4</td>
<td>5 ± 2</td>
<td>0.0121</td>
<td>0.1426</td>
<td>0.977*</td>
</tr>
<tr>
<td>⁶⁶⁶⁴Co</td>
<td>—</td>
<td>96 ± 26</td>
<td>82 ± 14</td>
<td>65 ± 10</td>
<td>49 ± 4</td>
<td>16 ± 4</td>
<td>11 ± 8</td>
<td>0.0156</td>
<td>0.1518</td>
<td>0.996***</td>
</tr>
<tr>
<td>⁶⁶⁶⁴Fe</td>
<td>51 ± 14</td>
<td>46 ± 8</td>
<td>38 ± 12</td>
<td>31 ± 8</td>
<td>20 ± 14</td>
<td>8 ± 3</td>
<td>6 ± 3</td>
<td>0.0146</td>
<td>0.1833</td>
<td>0.997***</td>
</tr>
<tr>
<td>⁶⁶⁶⁴Sr</td>
<td>96 ± 5</td>
<td>75 ± 7</td>
<td>—</td>
<td>48 ± 3</td>
<td>31 ± 6</td>
<td>9 ± 3</td>
<td>3 ± 0.6</td>
<td>0.0488</td>
<td>0.2106</td>
<td>0.977**</td>
</tr>
<tr>
<td>⁶⁶⁶⁴Ca</td>
<td>89 ± 5</td>
<td>68 ± 10</td>
<td>—</td>
<td>26 ± 1</td>
<td>1 ± 2</td>
<td>4 ± 0.4</td>
<td>3 ± 0.8</td>
<td>0.0289</td>
<td>0.3465</td>
<td>0.993**</td>
</tr>
<tr>
<td>⁶⁶⁶⁴Mn</td>
<td>94 ± 11</td>
<td>74 ± 7</td>
<td>—</td>
<td>18 ± 3</td>
<td>7 ± 3</td>
<td>0.9 ± 0.7</td>
<td>0.5 ± 0.3</td>
<td>0.0108</td>
<td>0.3706</td>
<td>0.997*</td>
</tr>
<tr>
<td>⁶⁶⁴Cd</td>
<td>91 ± 19</td>
<td>84 ± 12</td>
<td>—</td>
<td>28 ± 12</td>
<td>12 ± 10</td>
<td>5 ± 4</td>
<td>2.4 ± 2.1</td>
<td>0.0257</td>
<td>0.4386</td>
<td>0.986**</td>
</tr>
</tbody>
</table>

† Actual times of readings are 1:7 h, 3 h, 5 h, 8 h and 21:5 h.
‡ Actual times of readings are 1:2 h, 2:2 h, 4:75 h and 7 h.

***P < 0.001; **P < 0.01; *P < 0.05.
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100%  

Fig. 1. Percentage loss of copper, zinc, iron and manganese from the blood with time. Note that the curves are of the same basic shape but vary in the initial rate of loss and in the slow rate of 'excretion'. Standard deviations and data for other metals are given in Table 1.

Table 2. Half lives ($t_1/2$) of the fast and slow components of metal clearance from the blood and their concentration factor in the hepatopancreas and carapace

<table>
<thead>
<tr>
<th>Metal</th>
<th>$t_1/2$ fast (h)</th>
<th>$t_1/2$ slow (h)</th>
<th>Hepatopancreas</th>
<th>Carapace</th>
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<tbody>
<tr>
<td>Cu</td>
<td>9.00</td>
<td>25.8</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>4.86</td>
<td>57.4</td>
<td>35.0</td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>4.66</td>
<td>44.7</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>3.76</td>
<td>47.4</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>Sr</td>
<td>3.29</td>
<td>17.0</td>
<td>0.11</td>
<td>1.13</td>
</tr>
<tr>
<td>Ca</td>
<td>2.00</td>
<td>24.0</td>
<td>0.08</td>
<td>2.34</td>
</tr>
<tr>
<td>Mn</td>
<td>1.87</td>
<td>64.1</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Cd</td>
<td>1.58</td>
<td>27.0</td>
<td>33.0</td>
<td></td>
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metals. Thus, for class (b) metals the rate of loss from the blood was followed by a corresponding uptake into the hepatopancreas with relatively minor fluctuations in the other tissues. In order to standardize this data to take into account differences in the size of the animals and of the injection volume the accumulation of metal in the

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tissues has been expressed as a concentration factor (CF). This represents the ratio of the metal concentrated in a tissue compared with the expected concentration if the metal was distributed uniformly throughout the animal.

\[ \text{i.e. CF} = \frac{M_{\text{tissue, g dry wt tissue}^{-1}}}{M_{\text{inj, g dry wt animal}^{-1}}} \]

Values for the concentration factor are shown in Table 2.

**INTERPRETATION AND DISCUSSION**

The rate at which metals are cleared from the blood of the crayfish can be described as the sum of two exponential processes. It is not possible in most compartmental models to correlate directly the mathematically derived rate constants with the physiological processes associated with particular organs. It is likely, however, that in these experiments the slow component represents the rate of loss of metal from the animal via the antennal gland and the gills. The rate of urine production in the crayfish is 2.1 µg g⁻¹ h⁻¹ (Greenaway, 1972), which is too small to account for even this slow component of metal loss even if the concentration of metal in the urine is equal to that in the blood. The gills are known to be important as a major site of ion regulation and they probably also contribute to this component (Robertson, 1960; Parry, 1960; Bryan, 1960; Greenaway, 1972). The slow rate constant is, therefore, considered to contain these two components since it is clearly too small to account for the accumulation of most of the metals in the tissues of the body, which are therefore identified as at least a component of the fast rate constant.

The metals in the blood are bound to varying extents to the blood proteins (Andrews, 1967; Bryan, 1967; Greenaway, 1972). If this binding influences the
clearance from the blood there should be a correlation between it and the half life ($t_{1/2}$) of the fast component. It was shown by Irving & Williams (1953) that the stability constants of the first row transition metals form a characteristic series in the order $\text{Mn} < \text{Fe} < \text{Co} < \text{Ni} < \text{Cu} > \text{Zn}$, irrespective of the nature and number of co-ordinating ligands. A similar series occurs in the rates of clearance of metals from the blood (Fig. 1, Table 1) but it is shown more clearly if curve 'stripped' data is used since this eliminates the slow 'excretory' component. We have therefore plotted $t_{1/2}$ for the fast component of the blood clearance against the atomic number of these metals (Fig. 2). The results show a typical Irving-Williams series. If the half-life of this fast component of metal clearance is plotted against the stability constants of a number of ligands (e.g. ethylene diamine, Fig. 3) two aspects of this relationship are made clearer. Thus, the stronger the ligand-binding ability the larger the half-life of the metal in the blood. This suggests that the more firmly the metal is bound to the blood proteins the longer it is retained in the blood of the crayfish. It is also apparent, however, that cadmium and to a lesser extent zinc, are cleared from the blood faster than would be expected on this relationship.

It is known from a number of studies that calcium accumulates in the exoskeleton and stomach (including gastroliths) of crayfish but that most other metals are incorporated into the hepatopancreas (Bryan, 1967, 1968; Adams, Simkiss & Taylor, 1982; Lyon, Taylor & Simkiss, 1983). We have therefore plotted the concentration factor of these metals in the hepatopancreas against the $t_{1/2}$ of the fast component of metal loss from the blood (Fig. 4). This shows that the class (a) metals ($\text{Ca}^{2+}$ and $\text{Sr}^{2+}$) disappear rapidly from the blood but are not concentrated in the hepatopancreas. This is in keeping with their binding to oxygen donor ligands in the calcareous deposits of the body. Most of the metals ($\text{Mn}^{2+}$, $\text{Fe}^{2+}$, $\text{Co}^{2+}$, $\text{Cu}^{2+}$) become concentrated in the hepatopancreas and the extent to which this occurs increases the slower they are cleared from the blood (i.e. the larger the $t_{1/2}$). Two of the metals i.e. $\text{Cd}^{2+}$ and
Fig. 4. The concentration factor of metals in the hepatopancreas compared with $t_{1/2}$ of the fast component for metal loss from the blood.

Zn$^{2+}$ are, however, cleared rapidly from the blood and also concentrated to a very large extent in the hepatopancreas. It is apparent, therefore, that these metals are able to enter the hepatopancreas cells rapidly and become bound to ligands at that site. The competition for these metals by these intracellular binding molecules is so strong that this process contributes significantly to the removal of these metals from the blood and may account for the exceptional position of these metals in Fig. 3.

It is concluded, therefore, that the methods of interpreting metal-ligand binding can be applied to studies of the inorganic physiology of living systems. The very simple approaches adopted in this study suggest that class (a) metals (Ca$^{2+}$ and Sr$^{2+}$) are rapidly cleared from the blood onto oxygen donors in the exoskeleton and stomach. Most of the other metals are retained in the blood by binding to protein ligands. The stronger the binding characteristics of these proteins the longer the metal is retained in the blood and the greater the amount that is finally incorporated into the cells of the hepatopancreas. Two metals (Cd$^{2+}$ and Zn$^{2+}$) are cleared extremely rapidly from the blood apparently because they become associated with specific ligands in these cells. This would be in keeping with the occurrence of sulphur-donating groups on ligands that are known to occur in this tissue (Lyon et al. 1983). The approaches that have been adopted in this study suggest, therefore, that the principles of metal-ligand binding can be applied to whole physiological systems and that the results obtained provide useful insights into some of the biochemical processes that are involved.
REFERENCES


