THE EFFECTS OF THIOCYANATE ON THE INTRACELLULAR ION CONCENTRATIONS OF BRANCHIAL EPITHELIAL CELLS OF BROWN TROUT

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Summary
Exposure of brown trout (Salmo trutta) in fresh water to 0.15 mmol l\(^{-1}\) external thiocyanate for 1 h resulted in a significant decrease in chlorine concentration in the branchial mitochondria-rich (MR) cells from 37 mmol l\(^{-1}\) to 22 mmol l\(^{-1}\). The intracellular sodium concentration in these cells decreased by a similar amount, whilst the intracellular phosphorus concentration increased significantly. In contrast to the MR cells, 0.15 mmol l\(^{-1}\) external thiocyanate had no effect on the intracellular ion concentrations in the pavement epithelial cells. Thiocyanate is known to inhibit chloride uptake in a number of freshwater animals and therefore these data suggest that it is only the MR cells that are involved in the uptake of Cl\(^{-}\) in brown trout.

Key words: brown trout, Salmo trutta, gills, chloride, thiocyanate, X-ray microanalysis.

Introduction
The morphological location and the mechanisms of Na\(^{+}\) and Cl\(^{-}\) transport in freshwater teleosts have been the subjects of much debate. The majority of studies have favoured the mitochondria-rich (MR) or ‘chloride’ cells as the sites of Na\(^{+}\) and Cl\(^{-}\) uptake in fresh water (Laurent et al. 1985; Avella et al. 1987; Perry and Laurent, 1989): Na\(^{+}\) in exchange for H\(^{+}\) (or NH\(_{4}\)\(^{+}\)), Cl\(^{-}\) in exchange for HCO\(_{3}\)\(^{-}\) (for reviews, see Maetz, 1973; Maetz et al. 1976; Evans et al. 1982; McDonald et al. 1989). More recently, however, it has been suggested that Na\(^{+}\) uptake is via pavement epithelial (PE) or ‘respiratory’ cells (Goss et al. 1992a; Morgan et al. 1994) and that this uptake may occur passively via a Na\(^{+}\) channel, driven by a proton-pump-generated membrane potential (Lin and Randall, 1991, 1993; Perry and Laurent, 1993). In contrast, it has generally been assumed that the MR cells are the site of Cl\(^{-}\) uptake, although there is little direct evidence for this view.

The majority of studies on the location of Na\(^{+}\) and Cl\(^{-}\) transport systems have been based on correlations between cell morphology and gill ionic fluxes (Laurent et al. 1985; Avella et al. 1987; Perry and Laurent, 1989; Goss et al. 1992a,b). The complex structure and vascularization of the gills have, thus far, prevented the use of experimental techniques such as intracellular microelectrodes that can provide data on individual cells, while isolated, perfused gills do not survive well. There has, therefore, been little reliable, direct measurement of the intracellular ion concentrations in PE and MR cells. The use of low-temperature scanning electron microscopy (LTSEM) and X-ray microanalysis (XRMA), however, allows the concentrations of elements to be measured in individual PE and MR cells of brown trout (Salmo trutta L.) in situ. In a previous study using these techniques, Morgan et al. (1994) found that exposure of brown trout to changes in environmental [Na\(^{+}\)] suggested that the PE cells were the sites of Na\(^{+}\) uptake. Exposure of trout to external acetazolamide, and to changes in external [Cl\(^{-}\)], suggested that the MR cells were the sites of Cl\(^{-}\) uptake. The data leading to this latter conclusion were, however, less clear because some changes in PE cells similar to those in MR cells were found in response to these treatments. The present study was therefore undertaken to identify further the cellular location of Cl\(^{-}\) uptake by exposing brown trout to external thiocyanate.

Thiocyanate has been shown to inhibit active Cl\(^{-}\) uptake in a number of freshwater animals (Ehrenfeld, 1974; Dietz and Branton, 1979; Dietz and Hagar, 1990), and to changes in external [Cl\(^{-}\)], suggested that the MR cells were the sites of Cl\(^{-}\) uptake. The data leading to this latter conclusion were, however, less clear because some changes in PE cells similar to those in MR cells were found in response to these treatments. The present study was therefore undertaken to identify further the cellular location of Cl\(^{-}\) uptake by exposing brown trout to external thiocyanate.

Materials and methods
Brown trout (Salmo trutta L.) were transferred individually from the stock aquarium to a darkened Perspex chamber (51 capacity), supplied with flowing, aerated water at ambient temperature (8–13°C) and cooled by an outer water jacket, and
left to recover from any stresses incurred for 48 h. Control fish were removed and the intracellular concentrations of Na, P, Cl and K in the PE and MR cells were analysed as described below. The flow to the experimental chambers was then stopped and fish were exposed to 0.15 mmol l\(^{-1}\) external thiocyanate, by addition of KSCN to the water, for 1 h before necropsy.

XRMA of the PE and MR cells was carried out following the methods of Morgan et al. (1994) as follows. After the experimental treatment, the fish were killed by a blow to the head and individual gill filaments were removed and immediately slam-frozen on a polished copper block, cooled by partial immersion in liquid nitrogen to \(-196^\circ\text{C}\). Specimen preparation, from removal of the fish from the water to cryofixation, took between 1 and 4 min. Once frozen, the tissue and standard were planed (i.e. sections were cut from the surface and discarded) in a Reichert-Jung FC 4E cryoultramicrotome at \(-140^\circ\text{C}\) as a flat surface is required for consistent X-ray measurement. The specimens were transferred, without exposure to air, to the low-temperature stage (\(-160^\circ\text{C}\)) of a Jeol 840A scanning electron microscope, and were coated with aluminium by evaporation ready for XRMA.

XRMA of the PE and MR cells and standards was carried out using the thin window of a Link LZ5 detector and a Link 860 series 2 analyser under the following parameters: accelerating voltage, 15 kV; probe current, \(7 \times 10^{-11}\) A; analysis area, \(4 \mu\text{m}^2\); live time, 400 s.

Under these conditions, the mean depth of X-ray emission is 0.8 \(\mu\text{m}\) and 90% of the X-rays are emitted from above a depth of 1.2 \(\mu\text{m}\) (Oates and Potts, 1985).

The concentration, \(C\), of the four elements in the PE and MR cells in mmol l\(^{-1}\) tissue water was calculated from:

\[
C = \left(\frac{A}{B}\right)S,
\]

where \(A\) is the ratio of net:background X-ray counts of the element in the cell, \(B\) is the ratio of net:background X-ray counts of the element in the standard and \(S\) is the concentration of the element in the standard (mmol l\(^{-1}\)). The relationship between \(B\) and \(S\) is linear (Morgan et al. 1994). Ten analyses of the apical region of each cell type and of the standard were carried out on each of three or four specimens from separate fish for each experimental treatment. Cell types were distinguished on the basis of shape, size and location, and on the characteristic apical microridges of the PE cells (see Fig. 1 in Morgan et al. 1994). The mean concentrations of Na, Cl, K and P were calculated and compared statistically using a two-tailed t-test (\(P<0.05\)).

**Results and discussion**

Under control conditions (untreated fish), the concentrations of the elements in the PE and MR cells were similar to those in MR cells with the exception of chlorine, which was significantly higher in PE cells (Table 1). Exposure to 0.15 mmol l\(^{-1}\) external thiocyanate resulted in a significant decrease in

![Fig. 1. XRMA of (A) mitochondria-rich (MR) cells and (B) pavement epithelial (PE) cells of brown trout (Salmo trutta) exposed to 0.15 mmol l\(^{-1}\) external thiocyanate (SCN\(^{-}\)) for 1 h. * Indicates a value significantly different from the control; † indicates a value in MR cells significantly different from that in PE cells (\(P<0.05\)). Values are mean ± S.E.M., \(N=30–40\).](image)

<table>
<thead>
<tr>
<th>Element</th>
<th>Control (mmol l(^{-1}))</th>
<th>SCN(^{-}) (mmol l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>53±6</td>
<td>37±2*</td>
</tr>
<tr>
<td>Chlorine</td>
<td>61±5</td>
<td>45±3</td>
</tr>
<tr>
<td>Potassium</td>
<td>79±5</td>
<td>85±6</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>84±4</td>
<td>92±5</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M., \(N=30–40\).

*Indicates that the concentration of an element in MR cells is significantly different from that in PE cells (\(P<0.05\)).
35 mmol l\(^{-1}\)), whilst intracellular phosphorus concentration increased significantly. The potassium concentration in MR cells was not significantly affected by exposure to 0.15 mmol l\(^{-1}\) thiocyanate. In contrast to MR cells, exposure to 0.15 mmol l\(^{-1}\) thiocyanate had no significant effects on concentrations of the four elements in the PE cells (Fig. 1B).

There are a number of problems, both theoretical and practical, with the techniques used in this study. It is possible that a reduction in a branchial influx, in this case of Cl\(^-\), could result from a similar decrease in ion movement across both the apical and basal membranes of the cell with no net change in intracellular concentration. However, it can be hypothesized that there might be a delay or ‘lag’ following the manipulation of an apical ionic flux before the basal flux can respond, such that a disruption of the normal intracellular composition occurs. We believe that the changes in elemental concentrations in the branchial epithelial cells that we have measured in this study can be interpreted in this way. Cells that are involved in the transport of an ion across the epithelium and which would, therefore, have the highest fluxes of that ion, might be expected to show the biggest changes. The main technical problems of XRM have been discussed previously (Morgan et al. 1994), but it should be remembered that XRF measures total elemental concentrations; it cannot distinguish between ‘free’ and ‘bound’ forms.

Thiocyanate is known to behave chemically as a halogen; if a chloride salt is soluble then so is the thiocyanate salt, and vice versa. Thiocyanate is a powerful inhibitor of active Cl\(^-\) uptake in freshwater fish (Kerstetter and Kirschner, 1974; de Renzis, 1975) and in freshwater mussels (Dietz and Branton, 1979; Dietz and Hagar, 1990). A thiocyanate concentration of 1 mmol l\(^{-1}\) inhibited the branchial influx of Cl\(^-\) in the rainbow trout \(\textit{Oncorhynchus mykiss}\) by 67\% (Kerstetter and Kirschner, 1974), whilst branchial Cl\(^-\) influx in the goldfish \(\textit{Carassius auratus}\) was inhibited by approximately 80\% by 0.15 mmol l\(^{-1}\) external thiocyanate (de Renzis, 1975). This inhibition was reversible, and thiocyanate injected into the goldfish had no effect on Cl\(^-\) transport, and therefore de Renzis (1975) concluded that the inhibition occurred at the apical membrane of the ion-transporting cells.

Thiocyanate ions are much larger than chloride ions and so it is likely that the inhibition is physical. de Renzis (1975) found both competitive and non-competitive inhibition in that increased concentrations of thiocyanate caused a reduction in both the \(K_m\) and \(V_{\text{max}}\) of Cl\(^-\) uptake (de Renzis, 1975). Thiocyanate inhibition of Cl\(^-\) uptake in freshwater mussels \(\textit{Ligumia subrostrata}\) was of the competitive type (Dietz and Branton, 1979).

The effects of the exposure of brown trout to 0.15 mmol l\(^{-1}\) external thiocyanate strongly suggest that it is only the MR cells that are involved in the uptake of Cl\(^-\). In the present experiments, external thiocyanate caused a 40\% reduction in the chlorine concentration in the MR cells, which we believe occurred as a result of the apical inhibition of Cl\(^-\) transport into these cells. No reduction in intracellular chlorine following exposure to external thiocyanate was seen in the PE cells, suggesting that these cells are not involved in Cl\(^-\) transport.

Thiocyanate has no effect on sodium transport (Epstein et al. 1973; de Renzis, 1975); the decrease in sodium concentration in the MR cells may be a secondary effect as it is in the direction that would tend to maintain the electrical balance of the cell. The reason for the increase in phosphorus concentration in MR cells is not known. Similar rapid changes in phosphorus content of branchial cells were observed under conditions of low environmental sodium (Morgan et al. 1994) and have also been observed in human sweat glands (H. Elder, personal communication).

It is generally accepted that external Cl\(^-\) crosses the branchial epithelium of freshwater fish in exchange for intracellular HCO\(_3^-\); indeed, external thiocyanate also inhibits HCO\(_3^-\) excretion (de Renzis, 1975; Maetz et al. 1976). However, the precise mechanisms of the Cl\(^-\)/HCO\(_3^-\) exchange are uncertain. A number of studies have reported the presence of a membrane-bound anion-sensitive ATPase that may act as a Cl\(^-\) pump (de Renzis and Bornancin, 1977; Bornancin et al. 1980), but Battram (1987) found no evidence of anion-dependent ATPase activity and Van Amelsvoort et al. (1978) claimed that all so-called trout gill plasma membrane activity of anion-stimulated ATPase was in fact due to mitochondrial contamination. Recently, evidence has been found for a low-conductance Cl\(^-\) channel on the MR cells of the marine killifish \(\textit{Fundulus heteroclitus}\) (Marshall et al. 1994). In fresh water, however, such channels would require associated active export of a counter anion to achieve Cl\(^-\) uptake, as the electrochemical gradient would favour Cl\(^-\) secretion across the apical membrane of the gills. Unfortunately, the present results do not provide any evidence in preference of either a Cl\(^-\) pump or a Cl\(^-\) channel as the mechanism for Cl\(^-\) uptake across mitochondria-rich cells in fresh water: this is a potentially fruitful area for further research.

**References**


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