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RESEARCH ARTICLE

Transport of selenium across the plasma membrane of primary hepatocytes and enterocytes of rainbow trout

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SUMMARY

Transport of essential solutes across biological membranes is one of the fundamental characteristics of living cells. Although selenium is an essential micronutrient, little is known about the cellular mechanisms of chemical species-specific selenium transport in fish. We report here the kinetic and pharmacological transport characteristics of selenite and its thiol (glutathione and L-cysteine) derivatives in primary cultures of hepatocytes and isolated enterocytes of rainbow trout. Findings from the current study suggest an apparent low-affinity linear transport system for selenite in both cell types. However, we recorded high-affinity Hill kinetics (K_d=3.61±0.28 μmol l^{-1}) in enterocytes exposed to selenite in the presence of glutathione. The uptake of selenite in the presence of thiols was severalfold higher than uptake of selenite alone (at equimolar concentration) in both hepatocytes and enterocytes. Cellular accumulation of selenium was found to be energy independent. Interestingly, we observed a decrease in selenite transport with increasing pH, whereas selenite uptake increased with increasing pH in the presence glutathione in both cell types. The cellular uptake of selenite demonstrated a pronounced competitive interaction with a structurally similar compound, sulfite. The uptake of selenite as well as its thiol derivatives was found to be sensitive to the anion transport blocker DIDS, irrespective of the cell type. Inorganic mercury (Hg^{2+}) elicited an inhibition of selenite transport in both cell types, but augmented the transport of reduced forms of selenite in hepatocytes. Based on the substrate choice and comparable pharmacological properties, we advocate that multiple anion transport systems are probably involved in the cellular transport of selenite in fish.

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Key words: selenite, glutathione, L-cysteine, anion transporters, mercury, pH.

INTRODUCTION

Selenium is an essential micronutrient for all eukaryotes (Schwarz and Foltz, 1957). From an indispensable constituent of selenoproteins (Kryukov et al., 2003) to its role in cancer prevention (Rayman, 2005), selenium is proving to have diverse biological functions. At present, the physiological roles of many selenoproteins remain unknown. However, selenium can be toxic when its concentration marginally exceeds its nutritional requirement. Therefore, the maintenance of selenium homeostasis is of crucial physiological importance from both nutritional and toxicological perspectives.

Current knowledge on the mechanisms of cellular selenium transport systems is limited and much of the information comes from mammalian studies. Like its functionality, the chemical speciation of selenium (e.g. inorganic and organic forms in different oxidation states) in biological systems is also diverse. This adds to the complexity in understanding the cellular transport of selenium. The diverse speciation of selenium suggests that the cellular uptake of selenium is likely to occur *via* multiple membrane transporters, depending on its discrete chemical forms. Early work by McConnell and Cho suggested a passive transport of inorganic selenium (selenite) in the intestine of golden hamster (McConnell and Cho, 1965). Further investigation with human lymphocytes also indicated that the transport of selenite is a passive process and sensitive to sulfhydryl inhibitors (Porter et al., 1979). In human erythrocytes, anion exchanger 1 (AE1) has been suggested to be involved in selenite transport (Galanter et al., 1993; Haratake et al., 2009). However, in addition to selenite, AE1 can transport multiple other substrates (phosphate, sulfate and its target substrate bicarbonate) with high efficiency (Galanter et al., 1993). This raises an alternative possibility that phosphate, sulfate and other bicarbonate transporters may also transport selenite. A recent study suggested another type of molecular mimicry where the monocarboxylate transporter *Jen1P* can transport selenite in yeast (McDermott et al., 2010). In general, these findings indicate the possible involvement of multiple transporters in the cellular transport of selenite. Interestingly, it has been reported that selenite exhibits a lower transport rate than its reduced form(s) in yeast (Tarze et al., 2007) and in transformed keratinocytes (Ganyc and Self, 2008). Selenite can be effectively reduced by bioactive thiols (e.g. glutathione, cysteine) into several intermediates (e.g. selenotrisulfides, selenopersulfides and hydrogen selenide) (Ganther, 1971), which are also probably transported across the plasma membrane. However, it should be noted here that the specific transporters involved in the transport of these reduced intermediates of selenite are yet to be uncovered.

Currently, the mechanisms of selenite transport in fish, particularly at the cellular level, are largely unknown. It is interesting to study selenium transport in fish as their selenium requirement is

much higher (5–25 μg day^{-1} kg^{-1} body mass) (Janz, 2011) than that of humans (0.67 μg day^{-1} kg^{-1} body mass) (Brown and Arthur, 2001). This suggests that fish need to acquire and harness selenium efficiently to fulfill the requirement. Diet is the primary source of selenium in fish, and selenomethionine is considered to be the major chemical form in the natural fish diet (Maher et al., 2010). It has been suggested recently that the uptake of selenomethionine in fish intestine occurs *via* the methionine transporter(s) (Bakke et al., 2010). Interestingly, inorganic forms of selenium such as selenite constitute a substantial fraction (up to 15%) of the total selenium in the natural aquatic invertebrate prey species (Andrahennadi et al., 2007). In addition, detritivorous fish may absorb a significant amount of selenite as its concentration in the sediment can exceed over 20% of total selenium depending on the redox state of the system (Martin et al., 2011). Selenite can also be used as a supplement in commercial fish diet with no differences in growth and muscle selenium content when compared with its organic counterpart selenomethionine (Cotter et al., 2008). Following absorption in the intestine, selenite can be transported to the liver (the primary organ of selenium metabolism) *via* the hepatic portal circulation with or without first pass metabolism by enterocytes. The cellular systems involved in the transport of selenite and its interactions with other dietary/physiological components (e.g. thiols) need to be examined in fish. This will allow us to understand whether the selenium transport mechanisms in fish resemble those of mammals. Moreover, fundamental knowledge on the physiology of selenium transport in fish has important environmental implications as selenium has been categorized as one of the priority pollutants in aquatic ecosystems, and selenite is known to be the most toxic inorganic form of selenium to aquatic life (Hamilton, 2004). Thus, the present study was designed to investigate the kinetic and pharmacological properties of the transport of selenite and its reduced forms in isolated hepatocytes and enterocytes of a model teleost, rainbow trout (*Oncorhynchus mykiss*).

MATERIALS AND METHODS

Fish

Rainbow trout (*O. mykiss*, Walbaum 1792) weighing 200–300 g were obtained from the Saskatchewan Government Fish Farm, Lucky Lake, Saskatchewan. Fish were maintained in 1000 l flow-through aquaria receiving dechlorinated Saskatoon City water at a rate of 2 l min^{-1} under constant aeration. A photoperiod of 16 h light: 8 h dark and a water temperature of 15±1°C were maintained throughout the experimental period. The fish were fed once a day with Martin's commercial diet (Martin Mills Inc., Elmira, ON, Canada) at a ration of 2% of body mass. All fish were acclimated for at least 2 weeks prior to their use in the experiments. Fish used in an experiment were not fed on the day of the experiment. The measured concentration of selenium in the diet was 0.63±0.02 μg Se g^{-1} dry mass (N=3). The experimental protocol was in accordance with the Canadian Council for Animal Care Guidelines and was approved by the animal research ethics board at the University of Saskatchewan.

Chemicals

^{75}Se as selenous acid was purchased from the University of Missouri Research Reactor (UMRR), USA. Selenite, GSH (reduced glutathione), cysteine, collagenase, cell dissociation solution, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and dicyclohexylcarbodiimide (DCCD) were obtained from Sigma-Aldrich (Oakville, ON, Canada). High purity propionic acid, phloretin and acetazolamide were obtained from Acros Organics (Thermo Fisher Scientific, Ottawa, ON, Canada). Leibovitz's L-15 medium was purchased from Invitrogen (Hamilton, ON, Canada). All other chemicals used were of analytical grade and purchased from VWR (Mississauga, ON, Canada), unless mentioned otherwise.

Cell culture

Hepatocytes were isolated and cultured using standard methodology as outlined elsewhere (Misra et al., 2010). Cells were plated on 100 mm BD Primaria^{TM} plates (BD Biosciences, Mississauga, ON, Canada) to form a monolayer. All of the transport studies were carried out after 24 h of cell isolation. We used non-enzymatic cell dissociation solution (Sigma-Aldrich, St Louis, MO, USA) for harvesting cells to avoid cellular injury following the manufacturer's instructions. Cells were then washed twice in freshly prepared L-15 medium and kept at 15°C until used for experimentation.

Enterocytes were isolated following a previously established protocol in our laboratory (Kwong et al., 2010). Isolated cells were suspended in modified Cortland saline (mmol l^{-1}: NaCl 133.0, KCl 5.0, $CaCl_2$ 1.0, $MgSO_4$ 1.9, $NaHCO_3$ 1.9, NaH_2PO_4 2.9, glucose 5.5 and Hepes 10.0, pH 7.4). All the experiments with enterocytes were conducted within 4 h of isolation.

For both cell types, cell viability was measured by the Trypan Blue exclusion method using a Countess Automated Cell Counter (Invitrogen), and only cell suspensions showing ≥90% viability were used for the experiment. Cell viability was measured before and after each experimental treatment in order to ensure that there was no unintended cell death during the course of the experiment, and the results were discarded when >5% cell death was observed during any experimental treatment.

Kinetics experiments

The uptake of selenite as well as its reaction products with GSH or L-cysteine (both within the physiologically relevant concentration range) was examined. All flux experiments with hepatocytes were carried out either in L-15 medium or modified Hanks' medium (mmol l^{-1}: NaCl 136.9, KCl 5.4, $MgSO_4 \cdot 7H_2O$ 0.8, $Na_2HPO_4 \cdot 7H_2O$ 0.33, KH_2PO_4 0.44, Hepes 5.0, Na-Hepes 5.0 and $CaCl_2$ 1.5, pH 7.63), whereas the experiments with enterocytes were performed in modified Cortland saline. Both sets of experiments were conducted at 15°C using a thermostatically controlled water bath (Linberg Blue M, Thermo Fisher Scientific). A 400 μl sample of cell suspension containing 1×10^6 to 1.2×10^6 cells ml^{-1} was used in each experiment. In experiments where cells were transferred to a different medium, cells were first washed with the new medium then resuspended. Radiolabeled stock solution of selenite was prepared by adding ^{75}Se to cold selenite stock and diluted accordingly to achieve the target selenium exposure concentrations and specific activity (final activity: 14.9 μCi ml^{-1} for hepatocytes and 16.25 μCi ml^{-1} for enterocytes).

A synopsis of the different experimental treatments performed with each cell type is provided in Table 1. For hepatocytes, the concentration-dependent (0.0125–5 μmol l^{-1}) accumulation of [^{75}Se]selenite was examined in both the absence and presence of GSH (30 μmol l^{-1}) over an exposure period of 2–60 min. Similarly, we evaluated the accumulation of [^{75}Se]selenite (0.0125 μmol l^{-1}) in the absence and presence of L-cysteine (25–100 μmol l^{-1}) over 30 min of exposure. For enterocytes, we also examined the concentration-dependent (0.5–20 μmol l^{-1}) uptake of [^{75}Se]selenite in both the absence (for 30 min) and presence (for 10 min) of GSH (30 μmol l^{-1}). In addition, the time-dependent accumulation of [^{75}Se]selenite (3.5 μmol l^{-1}) was evaluated with (for 5–30 min) or

Table 1. Summary of the different experimental conditions under which [^{75}Se]selenite influx was evaluated in rainbow trout hepatocytes and enterocytes

Cell type	Treatment	[^{75}Se]selenite (hepatocytes: nmol l^{-1}) (enterocytes: μmol l^{-1})	GSH (μmol l^{-1})	Influx period (min)	Treatment effect results
Hepatocytes	[^{75}Se]selenite uptake kinetics	12.5–5000	±30	2–60[a,b]	Fig. 1A,B; Fig. 2A,B
	L-Cysteine (25–100 μmol l^{-1})	12.5	–	30	Fig. 3A,B
	NADPH (30 μmol l^{-1})	12.5	–	30	Fig. S1
	DIDS (0.0125–62.5 μmol l^{-1})	12.5	±30	10[a]/30[b]	Fig. 4A
	Sulfite (0.0125–125 μmol l^{-1})	12.5	–	30	Fig. 4B
	DCDD (0.0125–125 μmol l^{-1})	12.5	±30	10[a]/30[b]	Fig. S2A
	Orthovanadate (0.0125–125 μmol l^{-1})	12.5	±30	10[a]/30[b]	Fig. S2B
	pH (6.5–8.5)	12.5	±30	10[a]/30[b]	Fig. 5A,B
	Hg^{2+} (5–10 μmol l^{-1})	12.5	±30	10[a]/30[b]	Fig. 6A,B,E,F
	DIDS (62.5 μmol l^{-1}) ± Hg^{2+} (5 μmol l^{-1})	12.5	±30	10[a]/30[b]	Fig. 6A,B
	Phloretin (100–200 μmol l^{-1})	12.5	–	30	Fig. 7A
	Acetazolamide (100–200 μmol l^{-1})	12.5	–	30	Fig. 7A
	Sodium pyruvate (1–5 mmol l^{-1})	12.5	–	30	Fig. S3A
	Propionic acid (1–5 mmol l^{-1})	12.5	–	30	Fig. S3B
Enterocytes	[^{75}Se]selenite uptake kinetics	0.5–20	±30	10[a]/30[b]	Fig. 1C,D
	[^{75}Se]selenite uptake kinetics	3.5	±30	5–30[a]/5–60[b]	Fig. 2C,D
	L-Cysteine (30 μmol l^{-1})	0.5–3.5	–	30	Fig. 3C,D
	DIDS (50–100 μmol l^{-1})	3.5	±30	10[a]/30[b]	Fig. 4C,D
	Orthovanadate (50–100 μmol l^{-1})	3.5	±30	10[a]/30[b]	Fig. S2C,D
	pH (6.5–8.5)	3.5	±30	10[a]/30[b]	Fig. 5C,D
	Hg^{2+} (5–10 μmol l^{-1})	0.5–3.5	±30	10[a]/30[b]	Fig. 6C,D
	Phloretin (50–100 μmol l^{-1})	3.5	±30	10[a]/30[b]	Fig. 7B,C
	Sodium pyruvate (1–5 mmol l^{-1})	3.5	–	30	Fig. S3A

Note: a and b indicate the [^{75}Se]selenite influx period with and without 30 μmol l^{-1} of GSH, respectively.

without (for 5–60 min) GSH (30 μmol l^{-1}) in the exposure media. Similarly, we recorded the accumulation of [^{75}Se]selenite (0.5 and 3.5 μmol l^{-1}) in enterocytes in either the absence or presence of L-cysteine (30 μmol l^{-1}) over 30 min. For both cell types, the effect of GSH and L-cysteine on [^{75}Se]selenite influx was assessed following incubation of these thiol compounds with radiolabeled selenite for 10 min at 15°C. Furthermore, to examine whether the reducing equivalent or sulfhydryl group (GSH or L-cysteine) facilitates reduced selenium uptake, we examined the effect of NADPH (30 μmol l^{-1}, comparable to GSH and L-cysteine concentrations used) on the cellular [^{75}Se]selenite accumulation in hepatocytes under identical experimental conditions.

Each experimental treatment was run at least in triplicate, using cells isolated from individual fish, and the sample size (*N*) for the data corresponds to the number of individual fish used for each treatment. The uptake experiment was initiated by adding the required ^{75}Se stock volume at time zero. At the end of the exposure period, uptake was terminated using the respective ice-cold medium containing 100 μmol l^{-1} of selenite, and the cells were washed 3 times (centrifuged at 1000 ***g*** for 1 min each) at 4°C to remove any adsorbed ^{75}Se. The radioactivity of the cell pellet was counted by a Wallac 1480 Wizard 3″ Gamma Counter (Perkin Elmer, Waltham, MA, USA). The protein content of a reference cell pellet (equal cell number and treated alike except no radiolabelled ^{75}Se was added) was measured by the Bradford method (Bradford, 1976) using a commercial kit (Sigma-Aldrich) and bovine serum albumin (BSA) as the standard. The cellular accumulation (pmol mg^{-1} protein) of selenium was normalized with the protein content of the cell pellet and calculated as follows:

$$\text{Cellular accumulation} = \frac{\text{Count from the cell pellet}}{\text{Specific activity} \times \text{protein content}}, \quad (1)$$

where the count from the cell pellet is in c.p.m., the specific activity of selenium in the exposure medium is in c.p.m. pmol^{-1} and the protein content of the cell pellet is in mg. The rate of cellular selenium uptake (pmol mg^{-1} protein/time) was determined by dividing the cellular accumulation by exposure time.

Pharmacological experiments

We investigated the effects of several inhibitors or antagonists on the cellular accumulation of selenite, in both the presence and absence of GSH (30 μmol l^{-1}). The final [^{75}Se]selenite concentration in the exposure medium was 0.0125 μmol l^{-1} for hepatocytes (physiologically relevant) (Lorentzen et al., 1994) and 3.5 μmol l^{-1} for enterocytes in all experiments. In these experiments, cells were pre-incubated with the antagonists for 20 min at 15°C and subsequently exposed to [^{75}Se]selenite for 30 min (without GSH) or 10 min (with GSH). All of the antagonists used in this study (see below for details) were freshly prepared immediately prior to their use in the experiment. As most of the inhibitors are toxic, parallel quality control experiments were conducted to ensure that cell viability was not compromised during the course of the experiments. In each experiment, a control treatment was performed concurrently for a better comparison. Some of the experiments were carried out only with hepatocytes because of the much lower relative yield of enterocytes isolated from the trout intestine (see Table 1 for a summary of the various pharmacological treatments conducted with each cell type).

Effects of anion transport inhibitor

Based on a previous report that selenite may be transported *via* anion exchangers (Galanter et al., 1993), we examined the effects of DIDS (0.0125–62.5 μmol l^{-1} for hepatocytes and 50–100 μmol l^{-1} for enterocytes) on the accumulation of selenite and its reaction by-

products with GSH, as DIDS is a common inhibitor of cellular anion transporters.

Effects of an inhibitor of ATPase and a uncoupler of ATP synthesis

The majority of cellular anion transport processes are energy independent. Thus, we hypothesized that the selenite transport process is also energy independent, in both the presence and absence of GSH. Orthovanadate (a P-type ATPase inhibitor) and DCCD (an uncoupling agent of ATP synthesis) were used to examine whether the transport process is energy independent. The concentration of orthovanadate and DCCD in the exposure media ranged from 0.0125 to 125 μmol l^{-1} for hepatocytes and 50 to 100 μmol l^{-1} for enterocytes.

Effects of sulfite and monocarboxylates on selenite transport

We examined the inhibitory effect of a structurally similar compound sulfite (0.0125–125 μmol l^{-1}) on selenite transport in hepatocytes. To evaluate the possible role of a monocarboxylate transport system in selenite transport, we investigated the inhibitory effects of sodium pyruvate and propionic acid (each at 1–5 mmol l^{-1}) on selenite uptake in hepatocytes. The effect of sodium pyruvate (1–5 mmol l^{-1}) on selenite uptake in enterocytes was also examined. Experiments with propionic acid indicated a possible influence of pH on the cellular uptake of selenite (see Results for details). Therefore, we also studied the effects of pH (6.5–8.5) on selenite transport, in both the presence and absence of GSH. The pH of the exposure media was adjusted with 1 mol l^{-1} HCl or NaOH. It is also important to note here that pH is known to influence the speciation of inorganic selenium (Galanter et al., 1993) and the function of many solute transporters (e.g. monocarboxylate transporters) (Halestrap and Meredith, 2004).

Effects of mercury and aquaporin inhibitors

It is well known that mercury (Hg^{2+}) is a potential inhibitor of several carrier-mediated transport systems (Pritchard and Renfro, 1983). Thus, we studied the effects of the possible extracellular complexation of Hg^{2+} and selenium, and the interaction of Hg^{2+} with the transporter(s) involved in selenium transport, as well as a combination of both. For these experiments, cells were pre-incubated either with Hg^{2+} or with radiolabeled selenite stock spiked with Hg^{2+}, prior to the initiation of selenite influx studies with or without GSH. The concentration of Hg^{2+} in the exposure media ranged from 5 to 10 μmol l^{-1} (added as $HgCl_2$). We also used DIDS (62.5 μmol l^{-1}) along with Hg^{2+} to investigate whether a component of selenite uptake is DIDS insensitive.

As we recorded a pronounced inhibitory effect of Hg^{2+} on cellular [^{75}Se]selenite accumulation (see Results for details), the possible involvement of aquaporin in [^{75}Se]selenite transport was hypothesized, given that Hg^{2+} is a classic inhibitor of aquaporins. The capacity of aquaporin to transport other related metalloids such as arsenite, antimonite and silicon is in line with this possibility (Haddoub et al., 2009). Therefore, we examined the effects of two different aquaporin inhibitors, phloretin (100–200 μmol l^{-1} for hepatocytes and 50–100 μmol l^{-1} for enterocytes) and acetazolamide (100 and 200 μmol l^{-1}, used with hepatocytes only), in addition to propionic acid (described above).

Data analysis

All the statistical analyses and curve fittings were performed using SigmaPlot® (Version 11.0; Systat Software, Inc., Point Richmond, CA, USA). To determine the kinetic parameters of selenite transport, we used models that provided the best fit. To analyze the concentration-dependent uptake kinetics of reduced forms of selenium in enterocytes, the data were fitted with the following three-parameter sigmoid Hill equation:

$$f = \frac{B_{\max} \times X^h}{[K_d]^h + X^h}\ , \qquad (2)$$

where B_{max} is the maximum rate of uptake, K_d is the substrate concentration at which half the maximum velocity is attained and h is the Hill coefficient.

To analyze the inhibition kinetics, data were fitted with a ligand-binding model, with either one-site or two-site competition. For the one-site competition model, the following equation was used:

$$f = \min + (\max - \min) / [1 + 10^{(x - \log EC50)}]\ , \qquad (3)$$

and for two-site competition model, the following equation was used:

$$f = \min + \left(max - min\right) \times \left[\left\{ \frac{F_1}{\left(1 + 10^{\left(x - \log EC_{50[1]}\right)}\right)} \right\} + \left\{ \frac{\left(1 - F_1\right)}{\left(1 + 10^{\left(x - \log EC_{50[2]}\right)}\right)} \right\} \right]\ . \qquad (4)$$

Comparison among the different treatment groups was carried out using one-way ANOVA followed by Tukey's *post hoc* test or by a pair-wise *t*-test, as appropriate. All data are presented as means ± s.e.m. (N). $P \leq 0.05$ was considered to be significant when comparing different treatments.

RESULTS

Effects of the different chemical forms of selenium on the transport process

A direct comparison of the transport kinetics of [^{75}Se]selenite and its reduced forms between hepatocytes and enterocytes was not always possible as the range of exposure concentrations was different for each cell type. Data in Fig. 1A,B depict the dose dependency of [^{75}Se]selenite accumulation by hepatocytes in the absence and presence of GSH. Cellular accumulation showed a linear pattern in both cases. The linear transport profile was persistent up to a 50 μmol l^{-1} of [^{75}Se]selenite exposure dose (data not shown). In enterocytes, the rate of [^{75}Se]selenite uptake was linear over the concentration range tested (0.5–20 μmol l^{-1}) (Fig. 1C). However, the uptake followed saturable high-affinity Hill kinetics in the presence of GSH (Fig. 1D), and the maximum rate of uptake (B_{max}) was found to be 6.661±0.279 pmol mg^{-1} protein min^{-1}. The kinetic analysis of uptake suggested a two-site substrate interaction (Hill coefficient=1.911±0.23), with a substrate concentration corresponding to the half-maximal uptake rate (K_d) of 3.612±0.283 μmol l^{-1}.

The time-dependent selenium accumulation profile in hepatocytes reached saturation within 30 min in the absence of GSH (Fig. 2A), whereas saturation occurred at 10 min in the presence of GSH (Fig. 2B). The initial rate (first 2 min) of selenite uptake in the presence of GSH was about 8 times (5.66±0.66 pmol min^{-1} mg^{-1} protein) higher than that of selenite alone (0.69±0.13 pmol min^{-1} mg^{-1} protein). In enterocytes, [^{75}Se]selenite transport (3.5 μmol $l^{-1} < K_d$) did not attain saturation over the exposure period of 60 min (Fig. 2C). However, the time-dependent uptake profile indicated a saturable uptake over an exposure period of 10 min in the presence of GSH (Fig. 2D). One of the important findings of the present study was that cellular selenium accumulation, in both the absence and presence of GSH, was ~20 times higher in hepatocytes than in enterocytes at an equimolar selenite exposure concentration

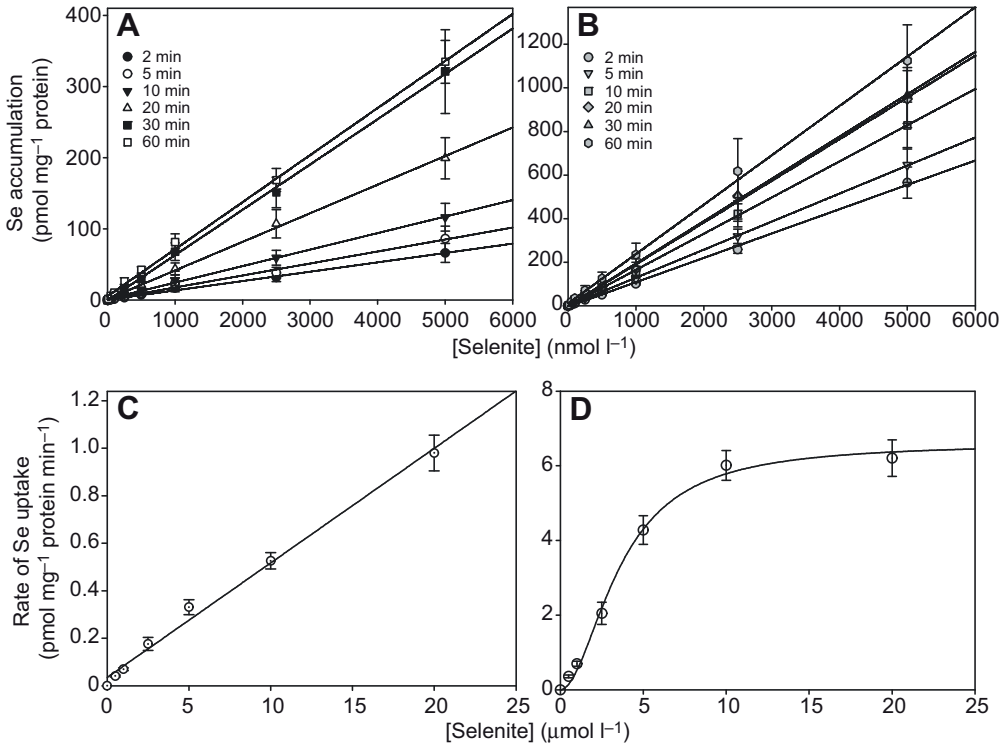

Fig. 1. (A,B) Concentration-dependent selenium accumulation in isolated hepatocytes, in either the absence (A) or presence (B) of 30 μmol l^{-1} GSH (reduced glutathione). Exposure times are as shown. (C,D) Kinetics of [^{75}Se]selenite transport in isolated enterocytes in the absence (C) and presence (D) of 30 μmol l^{-1} GSH. Exposure time for selenite and selenite+GSH was 30 and 10 min (for C and D), respectively. The values are expressed as means ± s.e.m. (N=5–6 independent measurements, each with cells isolated from an individual fish).

(0.5 μmol l^{-1}) for a specific exposure period (30 min in the absence of GSH and 10 min in the presence of GSH).

In the subsequent experiments, the effect of L-cysteine, another reducing agent, on [^{75}Se]selenite uptake was investigated. As L-15 medium is supplemented with amino acids, we conducted transport experiments in Hanks' medium as well as in L-15 medium to elucidate the effect of L-cysteine on [^{75}Se]selenite transport. The control experiment showed a higher uptake of [^{75}Se]selenite in L-15 medium than in Hanks' medium. However, when both exposure media were spiked with equimolar concentrations of L-cysteine, cellular selenium accumulation was comparable in the two media and significantly (P≤0.001) higher relative to that in the media not supplemented with L-cysteine (Fig. 3A,B). We also observed that selenium accumulation in hepatocytes (exposed in Hanks' medium) increased by ~10-fold in the presence of L-cysteine relative to the control, whereas the increase in accumulation was only ~5-fold in the presence of GSH (at a concentration comparable to L-cysteine). Similarly, selenium accumulation in enterocytes was ~18-fold higher in the presence of L-cysteine compared with the ~9-fold increase at an equimolar concentration of GSH (Fig. 3C,D), although it should to be noted that the selenite exposure concentration was 40- to 280-fold higher for enterocytes relative to that for hepatocytes.

In order to address whether it was the reducing equivalent or the sulfhydryl group (both GSH and L-cysteine contain a free –SH group) that facilitated uptake of reduced forms of selenium, we compared the effect of an equimolar concentration of NADPH with that of GSH and L-cysteine under identical experimental conditions. No effect of NADPH on [^{75}Se]selenite transport was observed in hepatocytes (see supplementary material Fig. S1), reinforcing the role of the interacting free thiol (–SH) group in efficient selenite reduction and the subsequent transport of reduced selenium species.

Energy independence of selenium transport

DCCD was found to be ineffective in blocking selenium transport in hepatocytes (see supplementary material Fig. S2A). Similarly, we also observed that orthovanadate did not inhibit the uptake of [^{75}Se]selenite or reduced forms of selenium in both hepatocytes and enterocytes (see supplementary material Fig. S2B–D). Together, these observations demonstrate the energy independence of inorganic selenium transport in both cell types.

Involvement of anion transport systems in selenium uptake

DIDS inhibited the accumulation of [^{75}Se]selenite in hepatocytes, in both the absence and presence of GSH, and the kinetic characterization indicated a single site interaction with DIDS (Fig. 4A). It has been proposed previously that anions such as sulfate and sulfite may be transported *via* a similar transport pathway to selenite in rat liver mitochondria (Crompton et al., 1974). We chose sulfite over sulfate in this study because of its much closer structural resemblance to selenite. Our results demonstrated a marked decrease of [^{75}Se]selenite accumulation in the presence of sulfite, and the kinetic characterization (ligand binding, two-site competition) suggested a competitive interaction between selenite and sulfite at two transport sites (Fig. 4B). In enterocytes, DIDS also inhibited the cellular accumulation of [^{75}Se]selenite, in both the absence and presence of GSH (Fig. 4C,D).

Differential effects of pH on selenium transport

No inhibition of [^{75}Se]selenite accumulation was recorded with up to 5 mmol l^{-1} pyruvate (sodium salt) in both hepatocytes and enterocytes (see supplementary material Fig. S3A). Similarly, 5 mmol l^{-1} propionic acid did not inhibit [^{75}Se]selenite accumulation in hepatocytes (supplementary material Fig. S3B) in pH-adjusted media (pH 7.63). However, we observed that the addition of propionic acid reduced the pH of L-15 medium, which was easily discernible from the obvious change of color (L-15 medium contains Phenol Red). In this pH-unadjusted medium, selenite transport was significantly (P≤0.001) higher than in the control (data not shown). This observation led us to deduce that pH probably plays an important role in selenite transport. Subsequent experiments at

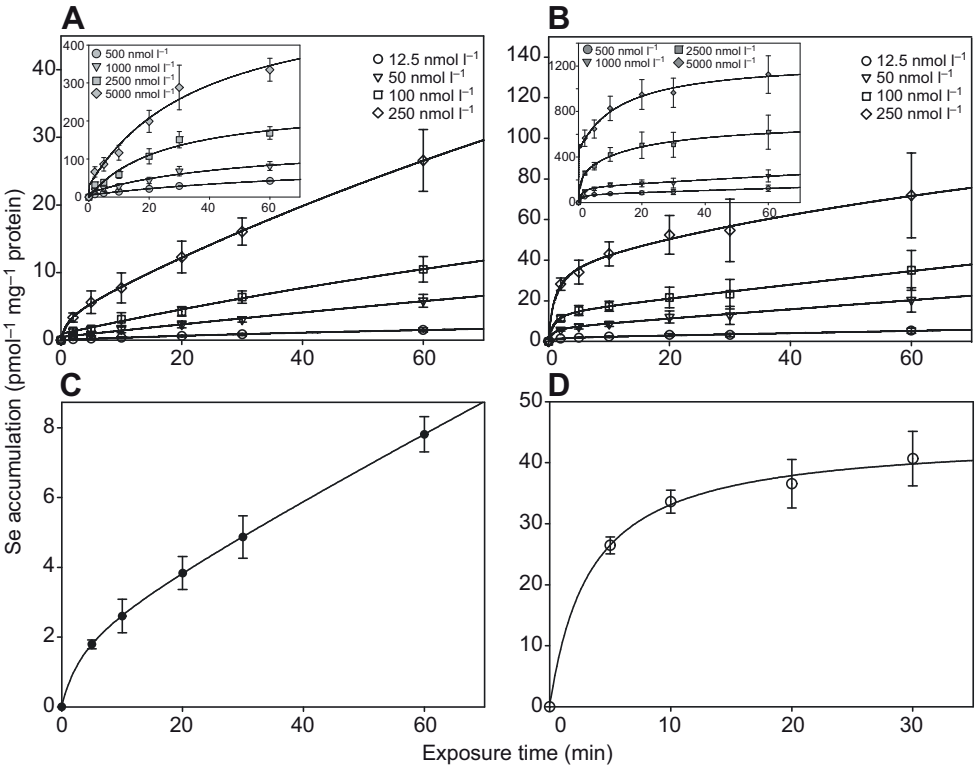

Fig. 2. Time-dependent selenium accumulation, in the absence (A,C) and presence (B,D) of 30 μmol l^{-1} GSH, in hepatocytes (A,B) and enterocytes (C,D). Insets in A and B show time-dependent selenium accumulation in hepatocytes at a relatively high concentration range of [^{75}Se]selenite exposure (500–5000 nmol l^{-1} *vs* 12.5–250 nmol l^{-1}). In C and D, the selenite concentration was 3.5 μmol l^{-1}. Values are expressed as means ± s.e.m. (*N*=5 independent measurements, each with cells isolated from an individual fish).

different pH indicated that selenium uptake is indeed pH dependent. With increasing pH, there was a decrease in [^{75}Se]selenite uptake in both cell types (Fig. 5A,C). Interestingly, we observed a completely opposite effect of pH in the presence of GSH as [^{75}Se]selenite uptake increased significantly in both cell types (Fig. 5B,D).

Extracellular component of selenium–mercury interaction

The effect of Hg^{2+} on cellular selenium transport was found to be quite complex in hepatocytes. Pre-exposure to Hg^{2+} significantly inhibited [^{75}Se]selenite uptake in L-15 medium and this inhibitory effect was greater than that of DIDS alone (Fig. 6A), indicating the possible existence of a DIDS-insensitive component of the uptake process. Interestingly, pre-exposure to Hg^{2+} significantly stimulated cellular [^{75}Se]selenite uptake in the presence of GSH (Fig. 6B). In contrast to hepatocytes, the effects of Hg^{2+} on cellular selenium transport in enterocytes in the presence and absence of GSH were consistent as pre-exposure to Hg^{2+} inhibited [^{75}Se]selenite uptake in both cases (Fig. 6C,D).

In addition, we aimed to understand the role of complexation of [^{75}Se]selenite with Hg^{2+} as well as the direct inhibition of transporter(s) by Hg^{2+}. This series of experiments was conducted with hepatocytes suspended in Hanks' medium to rule out any possible complex formation of Hg^{2+} with organic ligands present in L-15 medium. To delineate the role of complexation and thus limit the substrate availability, [^{75}Se]selenite (12.5 nmol l^{-1}) and mercury (10 μmol l^{-1}) were incubated together prior to the uptake study with unexposed hepatocytes. Significant inhibition of [^{75}Se]selenite uptake was observed (Fig. 6E, treatment I), indicating the potential effect of selenite and Hg^{2+} complexation. However, a direct inhibitory effect of Hg^{2+} on transporter function could not be ruled out as the molar concentration of Hg^{2+} was much higher than that of [^{75}Se]selenite. Nevertheless, when the hepatocytes were pre-exposed to Hg^{2+} and washed with an excess of media prior to the transport experiments, the inhibitory effect persisted (Fig. 6E, treatment II), indicating the direct interaction of Hg^{2+} with the transporter(s). The maximum inhibitory effect was observed in unwashed hepatocytes pre-exposed to Hg^{2+} (Fig. 6E, treatment III), but this was statistically not significant compared with the other two treatments (I and II). As observed previously in L-15 medium, the stimulatory effect of Hg^{2+} in the presence of GSH on cellular [^{75}Se]selenite uptake was consistent and remained in Hanks' medium (Fig. 6F, treatment II and III). However, when Hg^{2+} was pre-incubated with [^{75}Se]selenite-GSH mixture (treatment I), the cellular selenium uptake was found to be similar to that in the control.

Effects of aquaporin inhibitors on selenium transport

Both phloretin and acetazolamide elicited a modest but significant ($P \leq 0.05$) inhibitory effect on [^{75}Se]selenite uptake in hepatocytes (Fig. 7A). We only examined the effects of phloretin in enterocytes, and it did not inhibit [^{75}Se]selenite uptake in either the absence or presence of GSH (Fig. 7B,C).

DISCUSSION

To date, the knowledge of cellular selenium transport systems in fish is particularly limited. The results presented here provide novel information on the kinetic characteristics of selenium transport at a physiologically relevant concentration range in primary hepatocytes and enterocytes. [^{75}Se]selenite transport rate was linear in both cell types, which indicates the involvement of low-affinity, high-capacity transport systems. This is in agreement with previous findings in rat hepatocytes that exhibited a linear selenite uptake profile even up to supra-physiological levels (Park and Whanger, 1995). Similarly, our observation that selenite transport does not reach saturation in enterocytes is also in agreement with the transport of selenite in rat intestine (Wolffram et al., 1985). Nonetheless, in the presence of GSH, selenite uptake showed high-affinity (K_d=3.61±0.28 μmol l^{-1}) sigmoidal kinetics in enterocytes

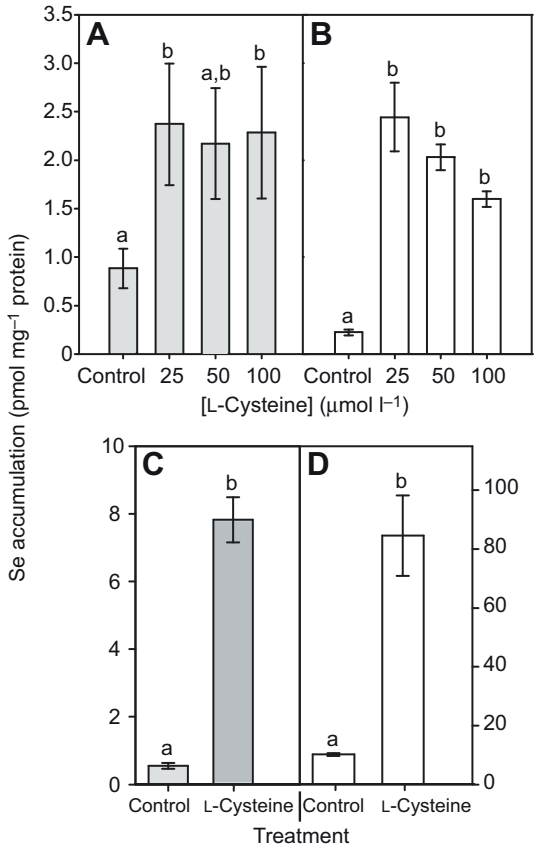

Fig. 3. (A,B) The effect of L-cysteine (25–100 μmol l^{-1}) on selenium accumulation in hepatocytes exposed to 12.5 nmol l^{-1} [^{75}Se]selenite for 30 min in L-15 medium (A) and Hanks' medium (B). (C,D) The effect of L-cysteine (30 μmol l^{-1}) on selenium accumulation in enterocytes exposed to 0.5 (C) and 3.5 μmol l^{-1} (D) [^{75}Se]selenite for 30 min. Data are presented as means ± s.e.m. (N=5 independent measurements, each with cells isolated from an individual fish). Mean values with different letters are significantly different (P<0.05). The statistical significance for data presented in A and B was evaluated by one-way ANOVA, whereas a pair-wise t-test was used for the data presented in C and D.

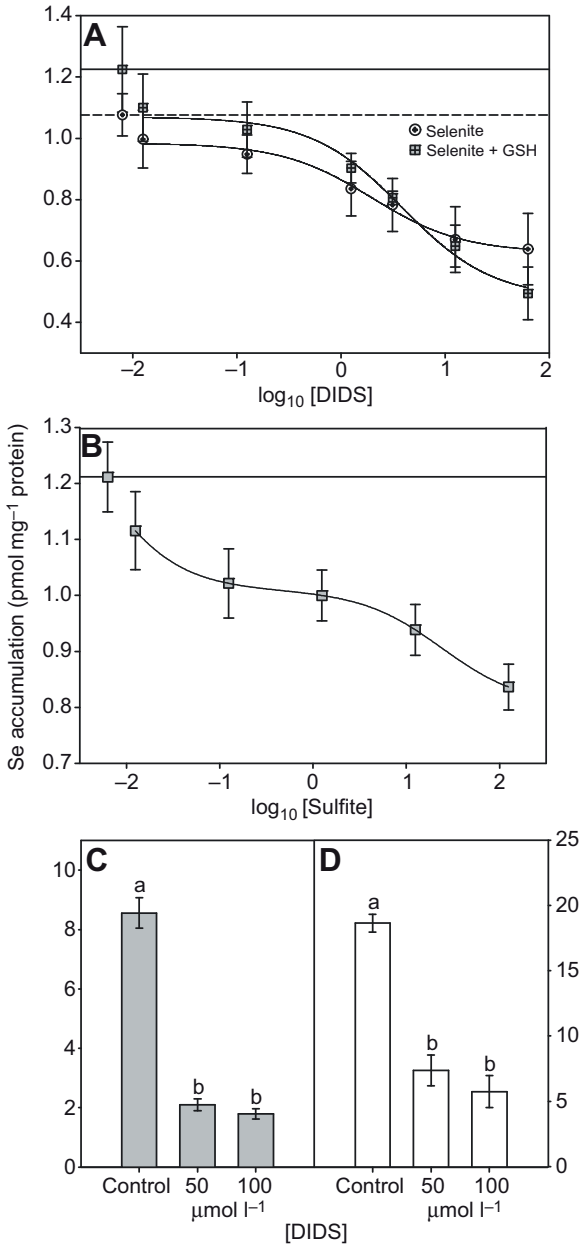

Fig. 4. (A) The inhibitory effect of DIDS on [^{75}Se]selenite (12.5 nmol l^{-1}) transport in hepatocytes, in either the absence or presence of 30 μmol l^{-1} GSH (N=5–8). The best fit of the inhibition data was achieved with the ligand-binding, one-site competition model (R^2=0.63 without GSH and R^2=0.38 with GSH, both values derived using the raw data). Dashed line indicates accumulation with selenite alone, solid line indicates accumulation with selenite+GSH. (B) The inhibitory effect of sulfite on [^{75}Se]selenite transport (12.5 nmol l^{-1}; N=5). The best fit of the inhibition data was obtained with the ligand-binding, two-site competition model (R^2=0.59, based on the raw data). Solid line indicates selenite uptake in the absence of sulfite. (C,D) The inhibitory effect of DIDS on [^{75}Se]selenite (3.5 μmol l^{-1}) transport in enterocytes, in the absence (C) and presence (D) of 30 μmol l^{-1} GSH (N=5–6). Data are presented as means ± s.e.m. (N represents the number of independent measurements, each with cells isolated from an individual fish). Mean values with different letters are significantly different (one-way ANOVA, P<0.05).

as opposed to the linear uptake rate in hepatocytes. Sigmoidal transport kinetics indicates positive co-operativity at transport sites in enterocytes. The Hill coefficient derived from the kinetics data was almost 2, signifying an interaction of the substrate with at least two transport sites. In such an allosterically regulated transport pathway, binding of the substrate to one site increases the binding affinity of the other. However, it should be pointed out that this derivation is based on fitting of the experimental data and remains inferential in the absence of direct evidence of multiple carrier systems.

We observed that incubation of selenite with GSH increased total selenium accumulation in trout hepatocytes and enterocytes, a phenomenon previously reported in isolated rat enterocytes (Anundi et al., 1984). In addition, we found that, like GSH, L-cysteine stimulated selenium uptake, but with a greater magnitude. Scharrer et al. previously observed similar effects of L-cysteine on selenium transport in rat intestine (Scharrer et al., 1992). Such an apparent increase in selenium uptake in the presence of different thiols seems to be attributable to either a differential reducing potential of the thiol moiety of the reducing agents or differences in transport properties of the intermediates generated as a result of the reaction of thiols with selenite. Examining the reaction of selenite with GSH and L-cysteine can partially explain such a dramatic increase in uptake. Under physiological pH, the reaction of selenite with GSH can form selenotrisulfide (GSSeSG), selenopersulfide (GSSeH),

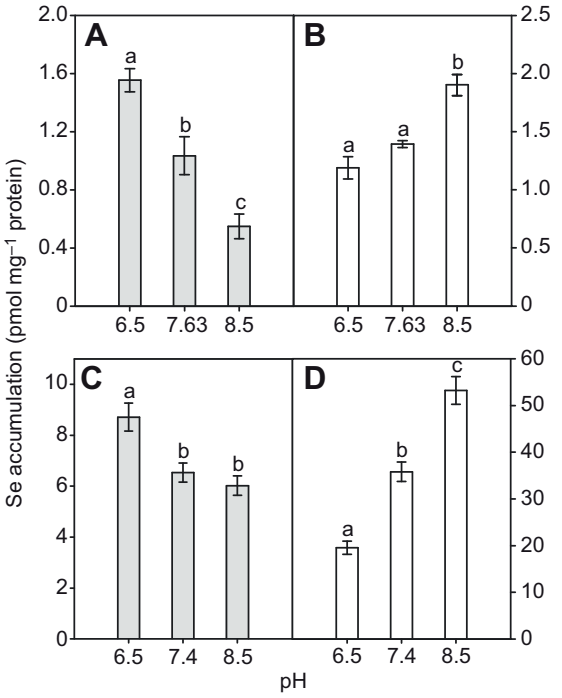

Fig. 5. (A,B) The effect of extracellular pH on the accumulation of selenium in hepatocytes exposed to 12.5 nmol l^{-1} [^{75}Se]selenite in the absence (A) and presence (B) of 30 μmol l^{-1} GSH. (C,D) The effect of extracellular pH on selenium accumulation in enterocytes exposed to 3.5 μmol l^{-1} [^{75}Se]selenite, in the absence (C) and presence (D) of 30 μmol l^{-1} GSH. Data are expressed as means ± s.e.m. (*N*=5), where *N* represents the number of independent measurements, each with cells isolated from an individual fish. Mean values with different letters are statistically significant (one-way ANOVA, $P<0.05$).

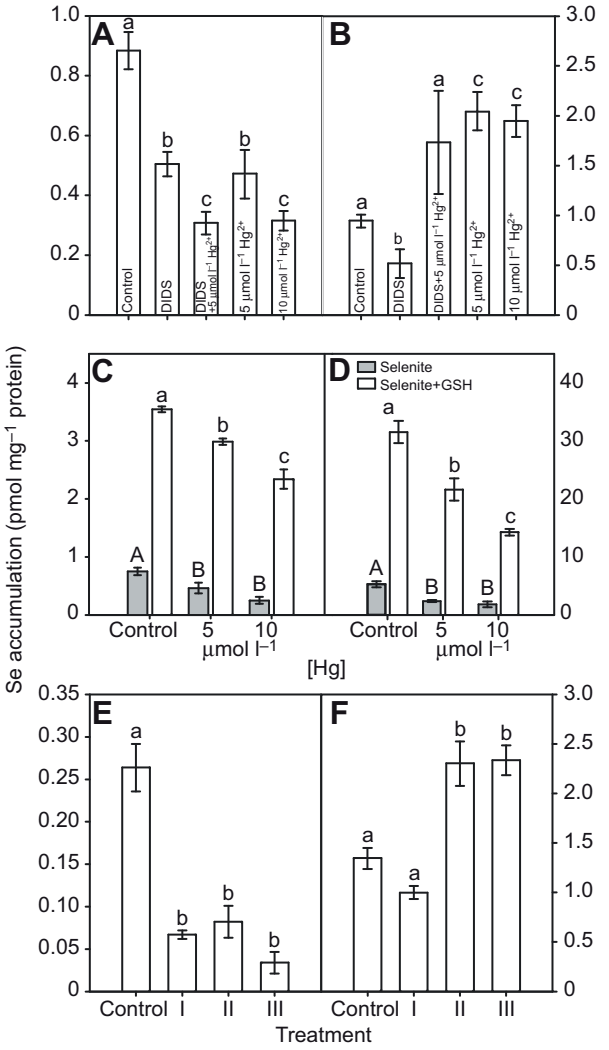

Fig. 6. (A,B) The effect of pre-exposure to inorganic mercury (Hg^{2+}) on [^{75}Se]selenite (12.5 nmol l^{-1}) transport, in the absence (A) or presence (B) of 30 μmol l^{-1} GSH, in hepatocytes exposed to [^{75}Se]selenite in L-15 medium with or without DIDS (62.5 μmol l^{-1}). (C,D) The effect of pre-exposure to inorganic mercury (Hg^{2+}) on [^{75}Se]selenite transport in enterocytes, in the absence or presence of 30 μmol l^{-1} GSH. Selenite concentration was 0.5 and 3.5 μmol l^{-1} in C and D, respectively. (E,F) The effects of different types of treatment with Hg^{2+} in hepatocytes exposed to [^{75}Se]selenite, without or with 30 μmol l^{-1} GSH, in Hanks' medium. Treatment I represents data when 12.5 nmol l^{-1} of [^{75}Se]selenite was incubated with 10 μmol l^{-1} Hg^{2+} for 20 min prior to the uptake experiment with unexposed cells; treatment II represents data when cells pre-exposed for 20 min to 10 μmol l^{-1} Hg^{2+} were thoroughly washed prior to the 12.5 nmol l^{-1} [^{75}Se]selenite uptake experiment; and treatment III represents data for the uptake of 12.5 nmol l^{-1} [^{75}Se]selenite in 10 μmol l^{-1} Hg^{2+} pre-exposed cells that were not washed. In experiments with GSH, Hg^{2+} was added 2 min after the mixing of [^{75}Se]selenite and GSH. Data are presented as means ± s.e.m. (*N*=5, where *N* represents the number of independent measurements, each with cells isolated from an individual fish). Mean values with different letters are significantly different (one-way ANOVA, $P<0.05$).

hydrogen selenide (HSe^- in solution) and colloidal elemental selenium (Se) (Ganther, 1971). We assume that a similar reaction scheme is also possible for L-cysteine with free thiol (–SH) group. At physiological pH, selenotrisulfide derivatives of GSH are very unstable compared with the persulfide derivatives, formed as a result of the reaction of GSH and colloidal elemental Se (Ganther, 1971). At present, the transport properties of selenopersulfides are not known. Nevertheless, it can be argued that their transport pathway may be similar to that of GSSG or cystine depending on selenopersulfides of GSH or cysteine, respectively, because of their structural similarity. It is important to note that the kinetics of GSH transport in isolated rat hepatocytes indicates a low-affinity sigmoidal influx system with a high K_m value of 2.36±0.26 mmol l^{-1} (Sze et al., 1993). However, testing this high K_m value for selenopersulfide in isolated cell systems is difficult because of the inherent high reactivity of this compound as well as its toxicity. In our experimental system with hepatocytes, the molar ratio of GSH to selenite varied between 6 and 2400, raising the possibility that HSe^- was the major form of selenium available for uptake. However, in our experiments with enterocytes this ratio was much lower and ranged from 1.5 to 60, which might have altered the speciation of selenium in the exposure media. It is unlikely that HSe^- was the only form of selenium involved in the uptake process in either cell type. Nevertheless, experimental evidence from the present study strongly suggests the presence of a high-affinity transport system for the reduced form(s) of selenium in enterocytes that is markedly different from that in hepatocytes. The relative contribution of different chemical forms of selenium, produced by the reaction of selenite with GSH or L-cysteine, remains an important question, and should be addressed in future investigations.

Selenium uptake was found to be energy independent irrespective of the chemical forms of selenium under investigation. Empirical

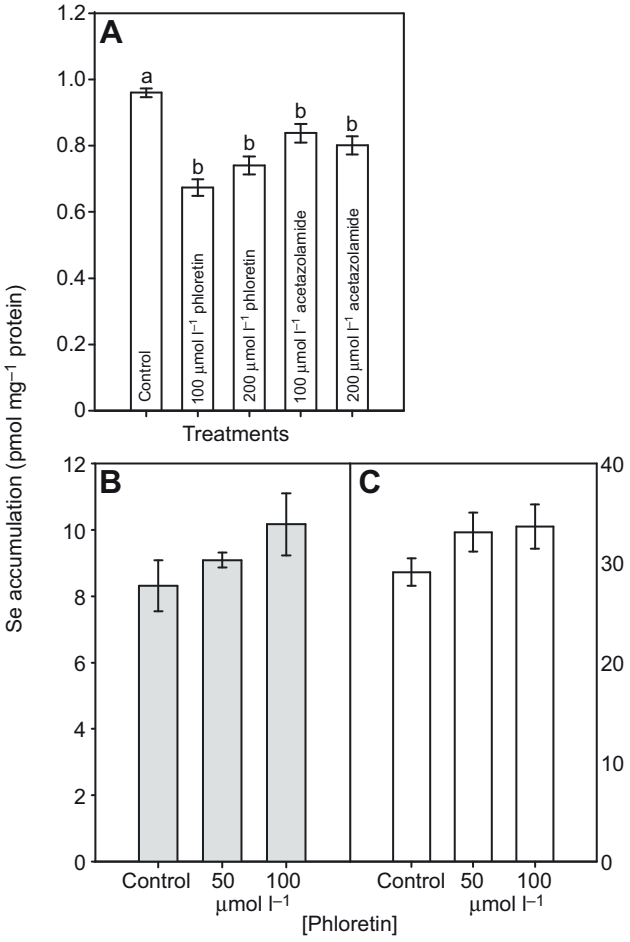

Fig. 7. (A) The effect of the aquaporin inhibitors phloretin and acetazolamide on selenium uptake in hepatocytes exposed to 12.5 nmol l^{-1} [^{75}Se]selenite. (B,C) The effect of phloretin on selenium uptake in enterocytes exposed to 3.5 µmol l^{-1} [^{75}Se]selenite, in the absence (B) and presence (C) of 30 µmol l^{-1} GSH. Data are presented as means ± s.e.m. (*N*=4, where *N* represents the number of independent measurements, each with cells isolated from an individual fish). Mean values with different letters are significantly different (one-way ANOVA, $P<0.05$).

evidence with human lymphocytes (Porter et al., 1979) and hamster intestine (McConnell and Cho, 1965) demonstrated this previously. The lack of energy dependency is consistent with the assumption of anion exchanger-mediated selenite transport, as such a facilitative transport system is driven by concentration gradients to mediate net bidirectional release or uptake of substrates. However, recent investigation in transformed keratinocytes indicates that selenite transport is an energy-dependent process (Ganyc and Self, 2008). In this context, it would be really interesting to understand why transformed cells like HaCat depend on energy for selenium transport and what energetic implications this might have on this type of cell when selenite is used as a chemotherapeutic agent.

Our finding that DIDS, an inhibitor of anion exchange, can block the uptake of selenite and its thiol derivative(s) is in agreement with a previous observation in keratinocytes (Ganyc and Self, 2008). However, we were not able to completely block selenium uptake with DIDS in both cell types. A previous study with erythrocytes indicated that about 90% of anion exchanger proteins were bound to DIDS (10 µmol l^{-1}) within 20 min of incubation (Ship et al., 1977). In the present experiment, we used a wide concentration range of DIDS (0.0125–100 µmol l^{-1}) with a similar incubation time, thus increasing the possibility of blocking the majority of anion transport activity. If the anion exchangers were the only membrane proteins involved in [^{75}Se]selenite transport, we should have observed a much higher degree of inhibition of cellular selenium accumulation at the highest exposure concentration of DIDS than we actually recorded. It is therefore conceivable that a component of cellular selenium (selenite and its thiol derivatives) transport might be DIDS insensitive, indicating the possible existence of alternative transport pathways.

Inhibition of [^{75}Se]selenite transport by the analogous oxyanion sulfite indicates the possible involvement of the transport pathway of the latter. Sulfate has also been found to effectively block selenite transport in mammalian erythrocytes (Galanter et al., 1993). Thus, the involvement of the SLC13 and SLC26 family of anion exchangers in cellular selenite transport is a real possibility, as the members of these transporter families are usually implicated in sulfate and sulfite transport (Labotka et al., 1989). It is important to note here that the expression of a Na^+-dependent sulfate transporter (SLC13A1) has been found in multiple tissues including the intestine of zebrafish (*Danio rerio*), and this transporter has also been suggested to be sensitive to anions like selenate and thiosulfate (Markovich et al., 2008). In addition, the sulfate/anion exchanger SLC26A1 has recently been cloned and characterized in rainbow trout kidney (Katoh et al., 2006), although its expression in other tissues has not yet been examined. In view of a previous report suggesting the Na^+ independence of selenite transport (Wolffram et al., 1986), the involvement of a Na^+-dependent sulfate transport pathway is unlikely. Instead, evaluating the role of proton-sensitive SLC26A1 and SLC26A3 in selenite transport in the liver and intestine would be of considerable interest based on their sensitivity to pH, respective tissue-specific expression and differential transport properties (Mount and Romero, 2004). This might provide some insight into the apparent differences in [^{75}Se]selenite transport characteristics between hepatocytes and enterocytes observed in the present study.

In our study, pH was found to strongly influence the cellular transport of selenite. Extracellular pH is an important determinant of selenite speciation. Selenite is in its deprotonated form at pH 7.6, whereas it is in its biselenite ($HSeO_3^-$) form at pH 6.5 (Olin et al., 2005). This speciation is highly dependent on the redox potential of the system and the presence or absence of interacting organic and inorganic ligands. While examining the effect of pH on selenium transport, two antithetical observations were documented based on whether GSH was added to the exposure media or not. Stimulation of [^{75}Se]selenite uptake at low pH closely resembles the characteristics of the anion exchanger family SLC26 involved in sulfite/sulfate transport (Galanter et al., 1993). Chloride-dependent anion exchangers (SLC4 family of transporters) may also be considered as important candidates at low pH, based on their sensitivity to DIDS and pH, and the structural likeliness of HCO_3^- and $HSeO_3^-$ (Romero et al., 2004). The molecular cloning and functional expression of SLC4 gene products have been reported in multiple fish species (Guizouarn et al., 2005; Shmukler et al., 2005). Likewise, the Cl^-/HCO_3^- exchanger SLC26A6 may also be involved in the transport of selenite, particularly in the enterocytes, given the mildly acidic nature and strong expression of this anion exchanger in fish gut (Grosell, 2011).

It is also noteworthy that pH sensitivity is one of the fundamental properties exhibited by monocarboxylate transporters (SLC16 family of transporters) (Halestrap and Meredith, 2004), and representatives of this family (MCT1–4) have recently been

implicated in selenite transport in yeast (McDermott et al., 2010). However, neither pyruvate nor propionic acid inhibited selenite transport in the present study. MCTs are known to be expressed in multiple tissues of fish (Ngan and Wang, 2009), and thus further evaluation of their involvement might be of particular interest, based on their wide spectrum of substrate choice and variable sensitivity to structurally similar compounds. The reason for the increase in the transport of the reduced form(s) of selenium with increasing pH in both cell types remains elusive without specific information on the effects of thiols on selenium speciation in our experimental systems. Thus, examining the uptake properties with homologous compounds is extremely difficult and would be purely speculative. To the best of our knowledge, this is the first report demonstrating the differential effect of pH on the cellular transport of selenite and its thiol derivative(s) in fish.

Much of the contemporary scientific interest in the selenium–mercury interaction has focused on the intracellular aspects, and the extracellular aspects of the selenium–mercury interaction have received very little attention. The extracellular interaction can occur either by complexation of selenium with Hg^{2+} before being transported into the cell or by interaction of Hg^{2+} with selenium transporters. We have clearly demonstrated the inhibitory effect of Hg^{2+} on [^{75}Se]selenite transport in both trout hepatocytes and enterocytes, which probably occurred for multiple reasons. The persistence of the inhibitory effect even after thorough washing of the cells suggests that the inhibition occurs at the transport site. The possible interaction of Hg^{2+} with a Hg^{2+}-sensitive amino acid residue (e.g. cysteine) in the pore-forming domain of the candidate transporter may effectively block [^{75}Se]selenite transport by preventing access to the pore, based on the notable similarity of ionic radii (4 Å) between Hg^{2+} and SeO_3^{2-}. Previous investigation with human lymphocytes suggested that sulfhydryl group-modifying agents (NEM, PCMB and iodoacetamide) can block selenite transport (Porter et al., 1979). In this context, it is important to note that the cysteine residue (Hg^{2+} interaction moiety) in the conserved STAS domain of sulfate transporters plays a pivotal role in its functionality (Rouached et al., 2005). Another possibility may be the limited availability of free selenite for transport during co-exposure of selenite with Hg^{2+} as a result of the formation of $HgSeO_3$ complex which has very low solubility (Zareh et al., 1995). Given the inhibitory effect of Hg^{2+} on selenite transport in both cell types, the stimulatory effect of Hg^{2+} on selenium transport in the presence of GSH in hepatocytes was not anticipated, and remains to be clarified. This is the first observation that Hg^{2+} augments a solute transport activity as opposed to its common inhibitory function on diverse types of transporters.

Our initial hypothesis on the possible role of aquaporins in selenite transport is constrained by the absence of any specific inhibitor of aquaporins. Similar to Hg^{2+}, acetazolamide and phloretin can also block anion transporter functionality (Pritchard and Renfro, 1983). In addition, propionic acid at higher concentrations can also inhibit aquaporin by inducing intracellular acidification (Tournaire-Roux et al., 2003). It is therefore difficult to either accept or rule out the possible role of aquaporin in the cellular transport of selenite. Moreover, other potential candidate transporters (e.g. SLC4, SLC26) show an isoform-specific degree of inhibition of their transport function by DIDS, phloretin and acetazolamide (Pritchard and Renfro, 1983; Halestrap and Meredith, 2004; Mount and Romero, 2004; Romero et al., 2004). Thus, the use of a pharmacological approach alone is not sufficient to ascertain the specific roles of these transporters in selenite transport.

Conclusion and future perspective

Collectively, the present study constitutes a major advance in our understanding of how selenite and its thiol derivatives are transported across the plasma membrane of enterocytes and hepatocytes in fish. We demonstrated that hepatocytes and enterocytes exhibit different selenite transport properties in the presence and absence of thiols. The present study also showed that the presence or absence of thiols determines the differential effects of extracellular pH on the cellular uptake of selenium. The apparent existence of selenium species-specific multiple transport mechanisms and their dependence on the proton electrochemical gradient will provide important impetus for future research on the nutritional and toxicological aspects of selenium in fish. As with previous investigations, the lack of understanding of chemical speciation of selenium in the systemic circulation limited our ability to select analogous compounds and examine the role of any possible pathway(s) involved in the uptake of thiol derivatives of selenite. A practical approach to resolve such issues relies on understanding the physiological speciation of selenium using techniques such as synchrotron-based X-ray spectroscopy. This will ultimately help to decipher the specific role of multiple transporters involved in the cellular transport of selenium.

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