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Interaction of selenite and arsenite on erythrocytes uptake of each metalloid: Possible mechanism mediated by extracellularly generated selenium-arsenic complexes

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Abstract

It has been demonstrated that selenium (Se) and arsenic (As) interact in living organisms. The elucidation of the interaction mechanisms of these toxic metalloids is believed to be of great importance for understanding their effects on humans and animals. The seleno-bis(S-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ has been identified as the major As metabolite present in the bile of rabbits and rats co-treated with selenite (iSeIV) and arsenite (iAsIII). Selenide (HSe^-), a reduced form of iSeIV, is a crucial component in the As-Se-GSH conjugate formation. This study aimed to shed light on HSe^- released from erythrocytes and propose a novel mechanism for the uptake of As and Se into erythrocytes via Se-As complexes, in addition to a previously reported mechanism for $[(GS)_2AsSe]^-$ formation. Erythrocytes from rats that had been fed As-depleted rodent chow for at least 7 weeks after weaning were used in the experiments. iAsIII and/or iSeIV were added to the erythrocyte suspension (final 10%) and incubated at 37°C for up to 180 min or 10 min. The uptake of both elements into the erythrocytes was assessed by measuring the attenuation of each element in the supernatant. HSe^- was detected by measuring the syn-(methyl,-methyl) bimane produced from the reaction of monochlorobimane with HSe^-. We observed that iAsIII was slightly taken up by erythrocytes. Furthermore, iSeIV was rapidly taken up by erythrocytes. Simultaneous exposure to both iSeIV and iAsIII increased Se and As uptake by erythrocytes. In addition, a temporal delay occurred in the uptake of both compounds. In conclusion, our study revealed that HSe^- release is more pronounced in selenite-treated erythrocytes and successfully proposed a novel mechanism for Se and As uptake.

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Introduction

Selenium (Se) is a sulfur analog that exists in nature in four oxidation states: selenate (iSeVI) (+6), selenite (iSeIV) (+4), elemental selenium (Se^0) (0), and selenide (HSe^-) (−2) [1]. Se is an essential trace element, yet it is well known that this substance is highly toxic, with a very

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narrow margin between safe and toxic levels. Se nutritional supply depends on its concentration in food. Both inorganic (iSeIV and iSeVI) and organic Se forms, such as selenocysteine and selenomethionine, can be used as dietary sources. Ingested Se compounds are reduced to HSe^-, which is subsequently used in selenoprotein synthesis [2]. Excess Se is methylated to form mono-, di-, or trimethyl-Se, which is then excreted from the body [3, 4].

Arsenic (As) is ubiquitous in the environment and has four oxidation states: arsenate (iAsV) (+5), arsenite (iAsIII) (+3), elemental As (0), and arsine (−3). It causes adverse health effects in humans, including cancer and multiorgan diseases. Millions of people worldwide suffer from chronic As poisoning [5]. However, As has been used to treat acute promyelocytic leukemia in humans [6]. Like Se, As is methylated in the body and excreted [7].

It has been established that Se and As interact in vivo. Understanding the interaction mechanisms of the toxic metalloids Se and As is crucial for elucidating their effects on animal and human health. The chemical properties of Se and As are analogous, and both are conjugated to glutathione (GSH) and methylated within the body [4, 8, 9]. It has been reported that co-treatment of rats with iSeIV and iAsIII resulted in the accumulation of both compounds in the blood and their excretion in the bile [10, 11]. The seleno-bis(S-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ has been identified as the major As metabolite present in the bile of rabbits and rats co-treated with iSeIV and iAsIII [12-14]. Erythrocytes have been identified as the production site of this excretory product in vivo [15, 16]. iSeIV is rapidly and selectively taken up by erythrocytes, where it is reduced to HSe^- [17, 18]. Following this reduction, HSe^- is transported to the plasma, where it selectively binds to albumin [18, 19]. The specific molecular mechanisms underlying the interaction between Se and As in erythrocytes remain unclear. Therefore, this study aimed to shed light on HSe^- released from erythrocytes and propose a mechanism for the erythrocyte uptake of As and Se via the Se-As complex, in addition to the $[(GS)_2AsSe]^-$ production mechanism proposed by Kaur et al. [16].

Materials and Methods

Chemicals and Reagents

Sodium arsenite ($NaAsO_2$; iAsIII: 97.0%), sodium selenite (Na_2SeO_3; iSeIV: 99.0%), monochlorobimane (MCB), syn-(methyl,-methyl) bimane, Trizma®base, and bovine serum albumin (BSA), were purchased from Sigma (St. Louis, MO, USA). 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was purchased from Tocris Biosciences (Bristol, UK). Standard solutions containing As and S (100 µg As/mL and 100 µg Se/mL; Accustandard®, New Haven, CT, USA) and rhodium (internal standard, 1.01 mg Rh/mL; Wako Pure Chemical Industries, Osaka, Japan) were used for total As and Se analysis. Working standard solutions (10 ng As/mL) were prepared daily using Milli-Q SP water (Yamato Millipore, Tokyo, Japan). GSH, nitric acid (HNO_3), hydrogen peroxide (H_2O_2), and other chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Animals and preparation of erythrocyte

Rats are one of the most tolerant animal species to As, and As is known to accumulate in rat erythrocytes following administration of iAs in the form of dimethylarsenical [20] [21]. It has also been reported that large amounts of As accumulate in erythrocytes after dimethylarsinic acid (DMAV) administration [22]. The commercial standard rodent chow (CE-2, Clea Japan Co., Tokyo, Japan) contained fishmeal as a protein source. Arsenobetaine was the major chemical form of As in the standard diet; however, small amounts of arsenocoline, iAsIII, DMAV, and iAsV were also detected [22]. To avoid As compound accumulation in red blood cell diets, pre-weaned rats were used. Male Sprague-Dawley rats (aged 2 weeks) and lactating female rats were obtained from Charles River Japan (Kanagawa, Japan). Following arrival, the lactating rats (with pups) were provided ad libitum access to As-depleted rodent chow (AIN-93G) [23], which used casein as the protein source instead of fish, purchased from Oriental Yeast Co. (Tokyo, Japan), along with sterilized deionized distilled water. Rats were maintained in an air-conditioned, clean room at 22–25°C, 50%–55% relative humidity, and a 12-h light-dark cycle. After 1 week of lactation, weanling male rats (3 weeks old) were separated from the dams and maintained on the same diet for at least 7 weeks until blood collection. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the National Institute for Environmental Studies (NIES).

Animals (>10 weeks old) were sacrificed by collecting whole blood from the abdominal aorta in heparin-coated evacuated blood collection tubes under anesthesia (sodium pentobarbital administration). Whole blood was centrifuged at 1,600 *g* for 10 min to remove the buffy coat and plasma. The erythrocytes were rinsed three times with tris-buffered saline (TBS, pH 7.4), and a 20% hematocrit erythrocyte suspension was prepared with TBS.

Procedure for total elemental analysis

A 100 µL of each sample was wet-digested with 0.6 mL nitric acid and 0.2 mL hydrogen peroxide at 90°C overnight and then at 130°C for 2 h in an aluminum block bath. The samples were diluted with Milli-Q water, and the concentrations of total As and Se were determined using the internal standard (Rh; m/z 103) method with inductively coupled argon plasma mass spectrometry (ICP-MS) (Agilent 7500cx or 8800; Agilent, Japan). The analytical method was validated by measuring As (7500cx (He reaction mode); m/z 75:$^{75}As^{+}$ or 8800 (O_2 reaction mode); m/z 91: $^{75}As^{16}O^{+}$) and Se (7500cx (H_2 reaction mode); m/z 78:$^{78}Se^{+}$ or 8800 (H_2 reaction mode); m/z 78: $^{78}Se^{+}$) concentrations in the reference samples(NIES CRM No. 18 human urine [NIES, Tsukuba, Japan, n = 5]). The certified and measured values for total As and Se in CRM No. 18 human urine were 0.137 and 0.133 As mg/L and 0.059 and 0.057 Se mg/L, respectively.

iAsIII and/or iSeIV uptake in erythrocytes with or without GSH or DIDS

iAsIII (final 10 µM) and/or iSeIV (final 10 µM) with or without GSH (final 5 mM) or DIDS (final 50 µM) were added to the erythrocyte suspension (final 10%) and incubated at 37°C for up to 180 or 10 min. Erythrocytes and supernatants were separated by centrifugation (8,000 *g*, 10 s). Each supernatant (n=3) was digested with HNO_3 and H_2O_2, and As and Se concentrations in the supernatant were determined using ICP-MS.

Se release from iSeIV-pre-treated erythrocytes and albumin and iAsIII effect on released Se reuptake

iSeIV (final 10 µM) was added to the erythrocyte suspension (final 10%) and incubated at 37°C for 10 min. The erythrocytes and supernatants were separated by centrifugation (2,000 *g*, 10 min) to prepare the iSeIV-pre-treated erythrocytes. The iSeIV-pre-treated erythrocytes were rinsed three times with TBS (pH 7.4), and a 20% hematocrit erythrocyte suspension was prepared with TBS. The untreated 10% and iSeIV-pre-treated erythrocytes used in this experiment contained 68.2 ± 3.68 µg/mL and 513 ± 18.4 µg/mL Se, respectively. BSA (final 50 mg/mL) and/or iAsIII (final 10 µM) were added to the erythrocyte suspension (final 10%) and incubated at 37°C for up to 10 min. TBS was added instead of BSA or iAsIII to serve as a control. The iSeIV-pre-treated erythrocytes and supernatants were separated by centrifugation (8,000 *g*, 10 s). Each supernatant (n=3) was digested with HNO_3 and H_2O_2, and As and Se concentrations in the supernatant were determined using ICP-MS. The same procedure was performed on erythrocyte suspensions that were not previously treated with iSeIV.

Detection of MCB reaction products with HSe^{-}

Untreated and iSeIV-pre-treated erythrocytes were suspended in TBS solution at a concentration of 10% and incubated at 37°C for 10 min. Erythrocytes and supernatants were separated by centrifugation (8,000 *g*, 10 s). The resulting supernatant was used for HSe^{-} measurement. HSe^{-} levels were measured according to the method described by Imai et al. [24]. Briefly, MCB (final 100 µM) was added to the supernatant and incubated at 37°C for 1 h. Then, an aliquot of 1 mL sample was extracted with 500 µL ethyl acetate. After dilution with 10 vol.% water, the extracted compounds were subjected to high-performance liquid chromatography (HPLC) analysis. The HPLC system (Shimadzu, Kyoto, Japan) consisted of a CTO-20AC column oven, LC-20AD solvent delivery module, DGU-20A3 degasser, RF-20A Fluorescence detector, and 7125 six-port injection valve with a 20 mL injection loop (Rheodyne, CA, USA). An aliquot of the sample (10 µL) was applied to a reversed-phase Luna C18(2) column (50 mm × 3.0 mm; GL Science, Tokyo, Japan) and eluted with 20 mM ammonium formate involving 50% acetonitrile as elution solution (flow rate of 1 mL/min). The samples were analyzed using a fluorometer at excitation and emission wavelengths of 380 and 480 nm, respectively.

Statistical analysis

Values are presented as the means ± SD of three samples. An unpaired multiple *t*-test was used to test the differences between the two means. To test the difference between multiple means, a two-way ANOVA followed by Tukey's multiple comparison test was performed. Statistical significance was set at $P < 0.05$. All analyses were performed using GraphPad Prism version 10.3.1 for Windows (GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com).

Results

Distribution of arsenic and selenium in erythrocytes, erythrocyte lysates, erythrocyte ghost, and supernatants of erythrocyte suspensions

The total recovery rates of Se and As in 10% erythrocytes and the supernatant were satisfactory, with values ranging from 90.6 to 99.0% (**Table S1**). The recovery rates of both elements from the erythrocyte membrane (ghosts) exhibited a range of 0.456 to 4.10%, with the majority of these elements present in the erythrocytes or the supernatant (**Table S1**). Consequently, in this study, the uptake of both elements into the erythrocytes was assessed based on the attenuation of each element in the supernatant.

Effects of simultaneous exposure to iAsIII on erythrocytes uptake of Se compounds, and vice-versa

In this study, consistent with previous reports [16, 17], iSeIV was rapidly taken up by erythrocytes (**Fig. S1a**). Except for iSeIV, Se compounds, such as iSeVI, selenomethionine, and selenocyanate, were infrequently incorporated into erythrocytes (**Fig. S1a**). Compared with the uptake levels observed for each Se compound, only iSeIV exhibited increased erythrocyte uptake in iAsIII presence (**Fig. S1a**). iAsIII was slightly taken up by erythrocytes; however, its uptake was elevated in iSeIV presence (**Fig. S1b**). In contrast, the coexistence of Se compounds other than iSeIV did not change iAsIII uptake compared with exposure to iAsIII alone (**Fig. S1b**).

GSH effect on erythrocyte uptake of As and Se compounds

Fig. 1 shows iAsIII (**Fig. 1a and b**) and iSeIV (**Fig. 1c and d**) uptake and the simultaneous exposure of iAsIII and iSeIV (**Fig. 1e and f**) in erythrocytes with or without GSH for up to 180 min in vitro. When exposed to iAsIII without GSH, iAsIII was slightly taken up by erythrocytes, and its uptake was inhibited in GSH presence. (**Fig. 1a and b**). Regarding iSeIV, approximately 30% and 67.3% of the added iSeIV was taken up by the erythrocytes immediately after the start of the reaction and within 5 min, respectively, in the absence of GSH (**Fig. 1c and d**). Se recovery from the supernatant decreased up to 5 min, increased slightly from 10 to 30 min, and then decreased gradually (**Fig. 1c**). GSH inhibited iSeIV uptake by the erythrocytes (**Fig. 1c and d**). However, approximately 40% of the added iSeIV was taken up by erythrocytes in GSH presence (**Fig. 1c and d**). In contrast, concurrent exposure to iAsIII and iSeIV in GSH presence significantly impeded the uptake of both elements by erythrocytes. (**Fig. 1e and f**). Compared with the uptake observed when each element was administered individually in GSH absence, iSeIV uptake increased, and iAsIII uptake was significantly enhanced when both elements were present concurrently (**Fig. 1**). Moreover, a time lag occurred between iAsIII and iSeIV uptake: iAsIII was taken up slightly later than iSeIV (**Fig. 1e and f**).

DIDS effect on erythrocyte uptake of As and Se compounds

iSeIV is transported into erythrocytes via band 3 anion transport proteins, also known as anion exchanger 1 (AE1) or band 3 [17]. As previously reported [16, 17], the iSeIV uptake was inhibited by DIDS (**Fig. 2b**). iAsIII uptake rate by erythrocytes did not change significantly in DIDS presence or absence (**Fig. 2a**). When erythrocytes were simultaneously exposed to iAsIII and iSeIV, the uptake of both elements was almost entirely suppressed in DIDS presence (**Fig. 2c**).

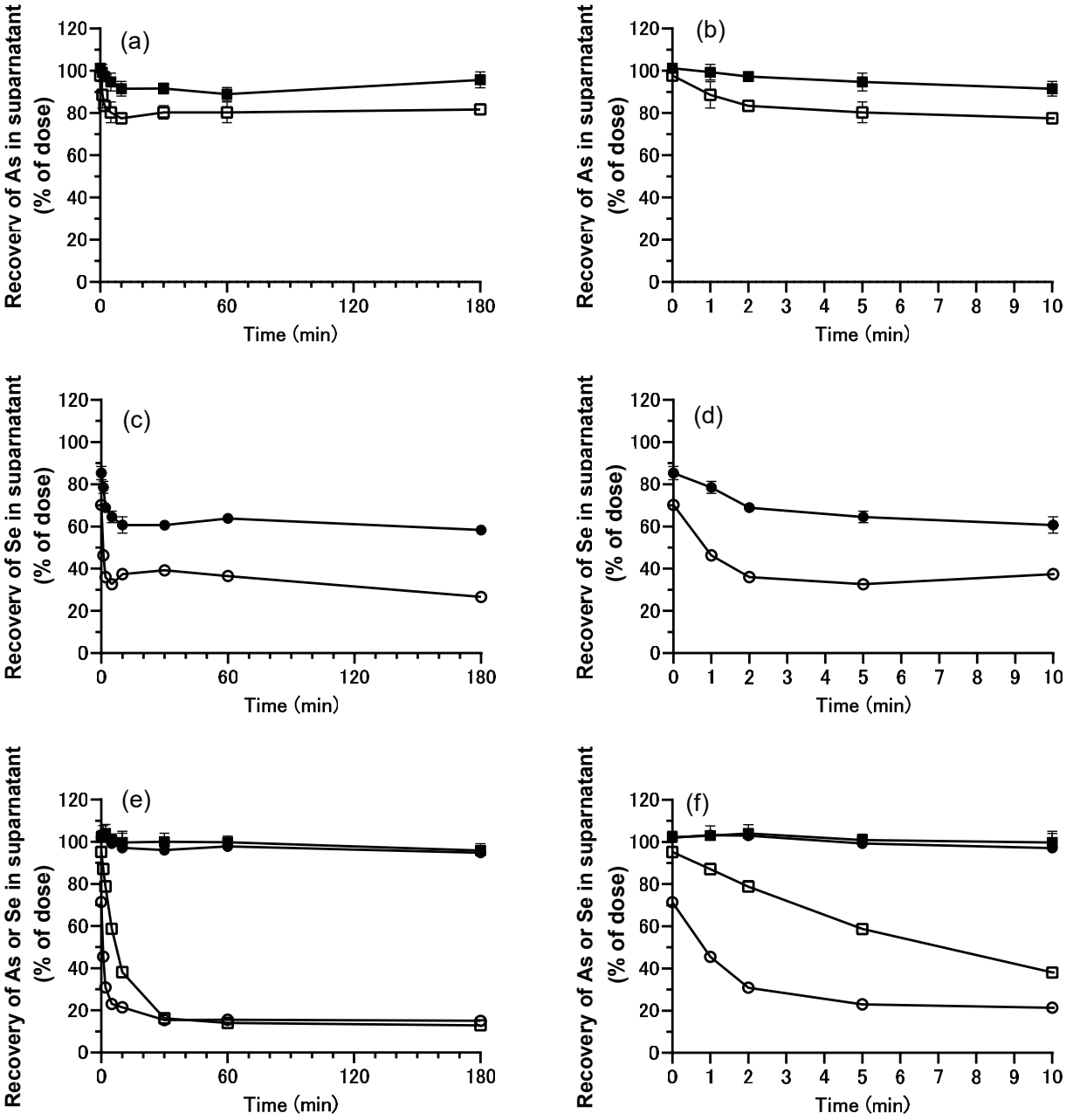

Fig. 1 **Time-course of changes in uptake of As and Se with or without GSH in erythrocytes up to (a, c, and e) 180 min, and (b, d, and f) results for a, c, and e up to 10 min.**

iAsIII (final 10 μM), iSeIV (final 10 μM), and iAsIII and iSeIV (final 10 μM of each) with (final 5 mM) or without GSH were added to the erythrocyte suspension (final 10%), and the mixtures were incubated at 37°C for up to 180 min. Erythrocytes and supernatants were separated by centrifugation (8,000 *g*, 10 s) and total As (m/z=75) and Se (m/z=78) contents in supernatant were determined by ICP-MS. (a and b) Changes in As recovery rate of supernatant in the presence or absence of 5 mM GSH. (c and d) Changes in Se recovery rate of supernatant in the presence or absence of 5 mM GSH. (e and f) Changes in As and Se recovery rate of supernatant in the presence or absence of 5 mM GSH. Vertical bars indicate As or Se recovery (% of dose) from the supernatant. Open squares and circles represent As and Se without GSH, respectively. Closed squares and circles represent As and Se with GSH, respectively. Values are presented as the mean ±SD (n = 3).

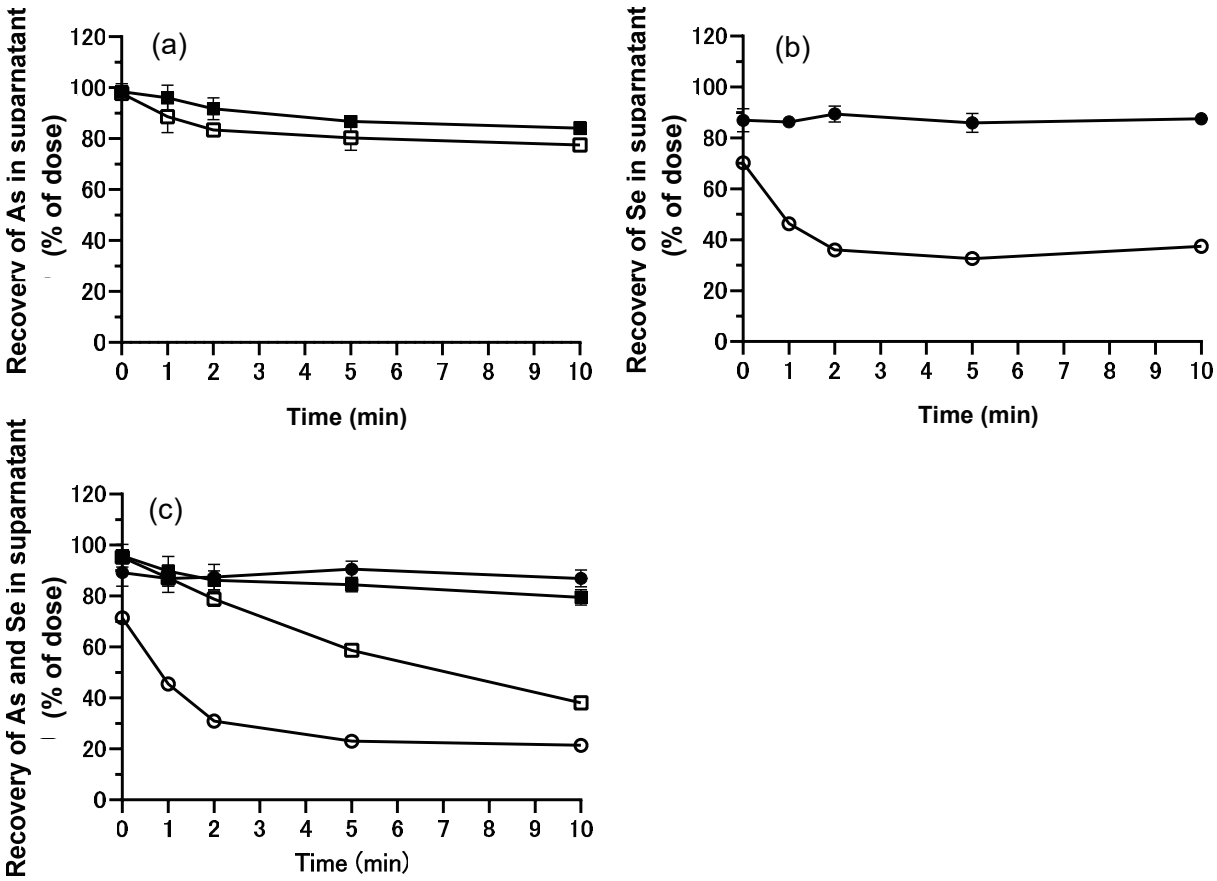

Fig. 2 **Time-course of changes in the uptake of As and Se with or without DIDS in erythrocytes up to 10 min. (a) iAsIII, (b) iSeIV, and (c) iAsIII and iSeIV**

iAsIII (final 10 μM), iSeIV (final 10 μM), and iAsIII and iSeIV (final 10 μM of each) with (final 50 μM) or without DIDS were added to the erythrocyte suspension (final 10%), and the mixtures were incubated at 37°C for up to 10 min. Erythrocytes and supernatants were separated by centrifugation (8,000 *g*, 10 s) and total As (m/z=75) and Se (m/z=78) contents in supernatant were determined by ICP-MS. (a) Changes in As recovery rate of supernatant in the presence or absence of 50 μM DIDS. (b) Changes in Se recovery rate of supernatant in the presence or absence of 50 μM DIDS. (c) Changes in As and Se recovery rate of supernatant in the presence or absence of 50 μM DIDS. Vertical bars indicate As or Se recovery (% of dose) from the supernatant. Open squares and circles, the same data as in **Fig 1**, represent As and Se without DIDS, respectively. Closed squares and circles represent As and Se with DIDS, respectively. Values are presented as the mean ±SD (n = 3).

Effect of albumin and iAsIII on released Se reuptake from iSeIV-pre-treated erythrocytes

iSeIV, which is taken up by erythrocytes and subsequently chemically modified within erythrocytes, selectively binds to albumin when released [17]. Consequently, plasma proteins, particularly albumin, may influence Se reuptake. iAsIII uptake into iSeIV-pre-treated erythrocytes was significantly enhanced compared with that in untreated erythrocytes (**Fig. 3a**). iAsIII uptake into erythrocytes was not affected by BSA, with or without iSeIV treatment (**Fig. 3a**). In BSA presence, Se recovery from the supernatant of the iSeIV-pre-treated erythrocyte suspensions was higher than that in the control group, whereas in iAsIII presence, the recovery was lower than that in the control group (**Fig. 3b**). In the presence of BSA and iAsIII, Se recovery from the supernatant of iSeIV-pre-treated erythrocyte suspension was lower than that observed in BSA presence and higher than that observed in iAsIII presence (**Fig. 3b**).

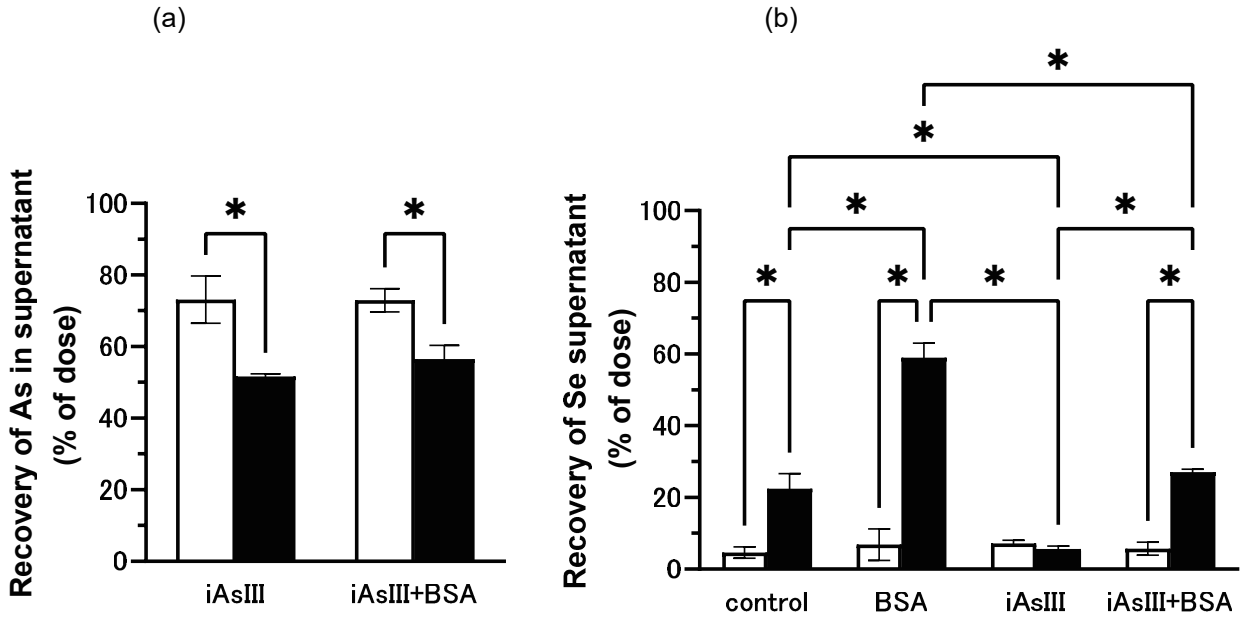

Fig. 3 **Recovery of (a) As with or without BSA and (b) effect of BSA and iAsIII on released Se uptake from untreated and iSeIV-pre-treated erythrocytes.** iAsIII (final 10 μM) was added to the untreated or iSeIV-pre-treated erythrocyte (final 10%) with or without BSA (final 50 mg/mL) and then incubated at 37°C for up to 10 min. As another experiment, BSA (final 50 mg/mL) and/or iAsIII (final 10 μM) were added to the untreated or iSeIV-pre-treated erythrocyte suspension (final 10%) and incubated at 37°C for up to 10 min. TBS was added instead of BSA or iAsIII to serve as a control. Erythrocytes and supernatants were separated by centrifugation (8,000 *g*, 10 s). Vertical bars indicate As or Se recovery (% of dose) from the supernatant. Open and closed bars represent untreated and iSeIV-pre-treated erythrocytes, respectively. Values are presented as the mean ±SD (n = 3). Asterisks indicate significant differences between the samples.

Identification of Se compounds released extracellularly from erythrocytes

In the bloodstream, iSeIV is rapidly taken up by erythrocytes and subsequently reduced to HSe^- [17-19]. Recently, a sensitive, selective, quantitative, and simple method for selenol and HSe^- detection has been reported [24]. This method involves MCB reaction, followed by the extraction of the product, syn(methyl, methyl)bimane (*syn*-MMB), using ethyl acetate. Under the conditions used in this study (see Materials and Methods for details), MCB peaks were detected at approximately 1.8, 2.8, and 2.2 min, in order of height. Syn-MMB was detected after approximately 2.2 min (**Fig. 4a**). Although an overlapping peak was observed at approximately 2.2 min, the MCB peak at this time accounts for approximately 8% of the total peak area. Consequently, it was concluded that this peak has a minimal impact on HSe^- detection. iSeIV did not react with MCB (**Fig. 4b**). The enhanced fluorescence intensity of *syn*-MMB in iSeIV-pre-treated erythrocytes, compared with untreated erythrocytes, indicated that HSe^- was released from the erythrocytes (**Fig. 4c and d**).

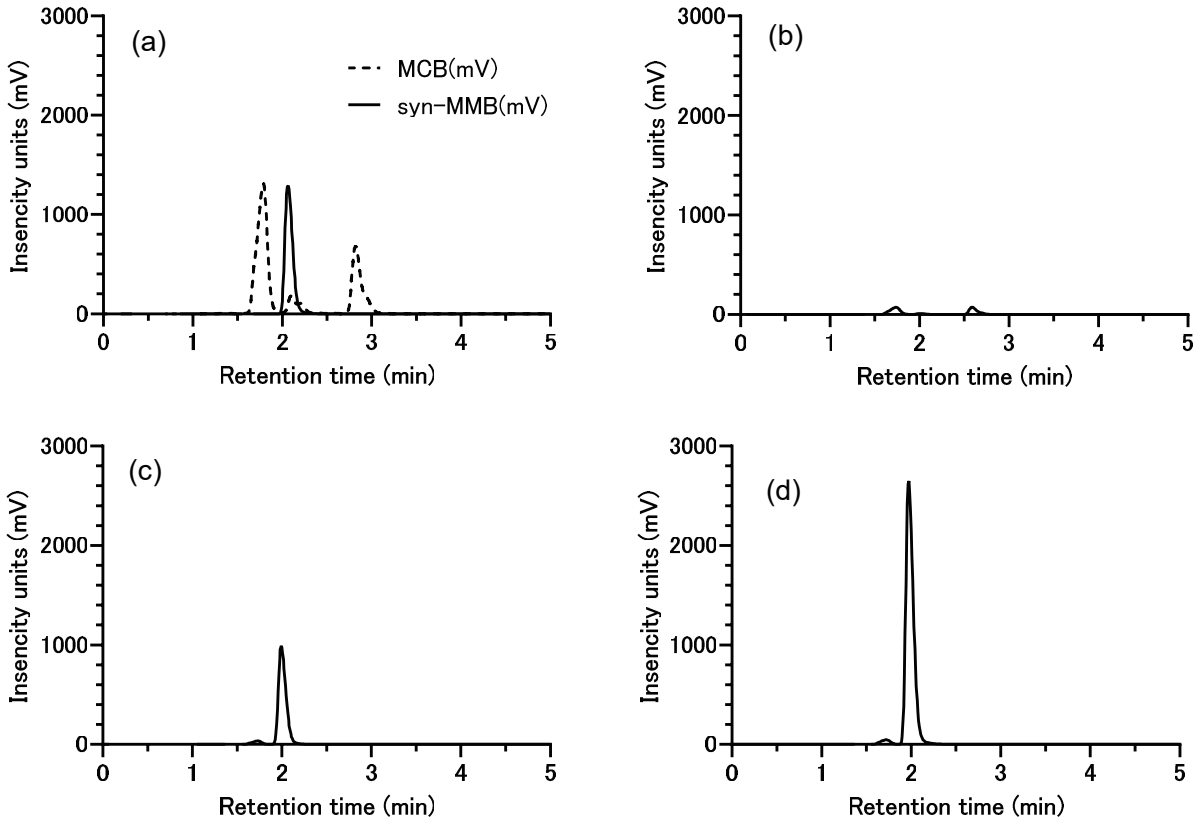

Fig. 4 **Elution profiles of MCB and syn-MMB from (a) standard solutions (0.1 mM MCB and 0.01 mM syn-MMB, respectively), samples reacted with (b) iSeIV, (c) untreated, or (d) iSeIV-pre-treated erythrocytes.**
A 10 µL sample was applied to a reversed-phase column and eluted with mobile phase (20 mM ammonium formate / acetonitrile = 50/50) at a flow rate of 1 mL min^{-1}. Vertical bars indicate intensity units (mV). The dashed line indicates MCB, and the solid line indicates syn-MMB, which is generated by the reaction of MCB with selenol or HSe^-.

Discussion

In this study, iAsIII was slightly taken up by erythrocytes (**Fig. 1a and b**). iAsIII uptake by human erythrocytes is partially inhibited by the aquaporin 3 (AQP3) inhibitors, indicating that AQP3 may play a significant role in iAsIII uptake [16]. AQP3 has been recognized as the primary channel responsible for glycerol transport in both human and rat erythrocytes [25, 26]. Consequently, it has been suggested that AQP3 plays a role in iAsIII uptake by erythrocytes in rats. As triglutathione ($As(GS)_3$) is generated non-enzymatically [8] and stabilized by GSH [27]. iAsIII uptake into the erythrocytes was inhibited in GSH presence (**Fig. 1a and b**). It is proposed that this phenomenon is the result of the non-enzymatic $As(GS)_3$ production as well as a shift in the equilibrium in favor of $As(GS)_3$ production by GSH.

Approximately 30% of the added iSeIV was taken up by the erythrocytes immediately after the start of the reaction (**Fig. 1c and d**). The addition of the sample to the erythrocyte suspension and the subsequent separation of the supernatant required approximately 30 s. The results of this study indicate that iSeIV uptake into erythrocytes occurred during this time, i.e., iSeIV was rapidly taken up by the erythrocytes. iSeIV is transported into erythrocytes through band 3 anion transport proteins [16, 17], and its uptake was inhibited by DIDS (**Fig. 2b**). In contrast, iAsIII

uptake by erythrocytes was unaffected by DIDS (**Fig. 2a**). Se recovery in the supernatant exhibited a biphasic pattern, initially decreasing for up to 5 min, then increasing slightly from 10 to 30 min, and subsequently decreasing gradually (**Fig. 1c**), suggesting that Se taken up by erythrocytes is released outside the erythrocytes and then reabsorbed. iSeIV uptake into the erythrocytes was inhibited in GSH presence (**Fig. 1c and d**). Painter first reported that iSeIV reacts with thiol-containing compounds in vitro to produce RSSeSR′ (H_2SeO_3+4RSH→RSSeSR+RSSR+$3H_2O$) [28]. Subsequently, a series of experiments were conducted to investigate the reaction between iSeIV and GSH [29]. The results demonstrated that the redox reaction of GSH with iSeIV occurs according to the following equation: $2H^+$+4GSH+SeO_3^{2-}→GSSG+GSSeSG+$3H_2O$ [30].

GSSeSG has been proposed as an intermediate and major inorganic Se metabolite [31]. Subsequent GSH reduction produces GSH selenopersulfide ($GSSe^-$) from GSSeSG, which can be readily decomposed to produce Se^0 as the end product or further reduced to HSe^- in the presence of a large excess of GSH [30, 31]. It has been suggested that the production of these Se compounds reduces iSeIV uptake by erythrocytes.

Consistent with previous reports [16], simultaneous exposure to both elements increased iSeIV uptake and significantly enhanced iAsIII uptake into erythrocytes compared with the uptake observed when each element was exposed individually in GSH absence (**Fig. 1**). A temporal delay occurred in iAsIII and iSeIV uptake, with iAsIII being absorbed marginally later than iSeIV (**Fig. 1e and f**). We concentrated on this aspect and predicted the pathway through which $[AsSe(OH)_2]^-$ is generated via the reaction between HSe^- and iAsIII outside of erythrocytes (**Fig. 5b**). The untreated 10% erythrocytes used in this experiment contained 68.2 ± 3.68 μg/mL Se, and the iSeIV-pre-treated erythrocytes contained 513 ± 18.4 μg/mL Se. In this study, HSe^- release from untreated erythrocytes was observed; however, the release was significantly more pronounced in erythrocytes treated with iSeIV (**Fig. 4c and d**). HSe^- is produced by GSH reduction in erythrocytes, selectively binds to albumin, and is transported to the liver [17, 18, 32]. Se release from iSeIV-pre-treated erythrocytes was markedly enhanced in BSA presence compared with that in its absence (**Fig. 3b**). HSe^- was released from erythrocytes and rapidly re-uptaken into the erythrocytes. In the presence of albumin, most of the released Se was rapidly bound to albumin, suggesting that reabsorption into erythrocytes was inhibited (**Fig. 5b**). Conversely, Se release from iSeIV-pre-treated erythrocytes in iAsIII presence was markedly lower than in its absence (**Fig. 3b**). In contrast, iAsIII uptake was significantly increased in iSeIV-pre-treated erythrocytes compared with that in untreated cells (**Fig. 3a**). This phenomenon indicates that HSe^- reacts with iAsIII, resulting in the formation of an As-Se compound that is subsequently taken up by erythrocytes, and then conjugated with GSH to produce $[(GS)_2AsSe]^-$. Furthermore, the fact that the uptake of Se and As into erythrocytes when iSeIV and iAsIII were added simultaneously was inhibited by GSH (**Fig.1e and f**) suggests that $[AsSe(OH)_2]^-$ generated outside erythrocytes reacted with excess GSH outside erythrocytes to generate $[(GS)_2AsSe]^-$.

Harper et al. [33] used density functional theory (DFT) and solvent-assisted proton exchange (SAPE) to model the reaction mechanism of iAsIII and iSeIV with thiols and the formation of the As-Se species $(RS)_2AsSeH$. Based on the DFT-SAPE results, the reaction of HSe^- with $As(SR)_2(OH)$ was reported to be the most favorable mechanism for As–Se bond formation [33]. It has been reported that the pathway where HSe^- attacks iAsIII to form intermediate $As(OH)_2SeH$, followed by RSH as a nucleophile to form As $(RS)_2SeH$, requires more energy and depends on the rates of iAsIII consumption and RSSeH and HSe^- formation [33]. The limitation of this study is the inability to identify As-Se compounds, such as $[AsSe(OH)_2]^-$ and $[(GS)_2AsSe]^-$ (**Fig. 5b**). We have attempted to detect As-Se complexes but have not succeeded yet.

In conclusion, it is highly likely that the pathway presented in **Fig. 5a** is the main pathway for $[(GS)_2AsSe]^-$ formation; however, the possibility that the pathway presented in **Fig. 5b** is an alternative pathway cannot be ruled out. Further research is needed to clarify these issues, including the isolation of As-Se compounds, which are thought to be reaction intermediates and kinetic studies.

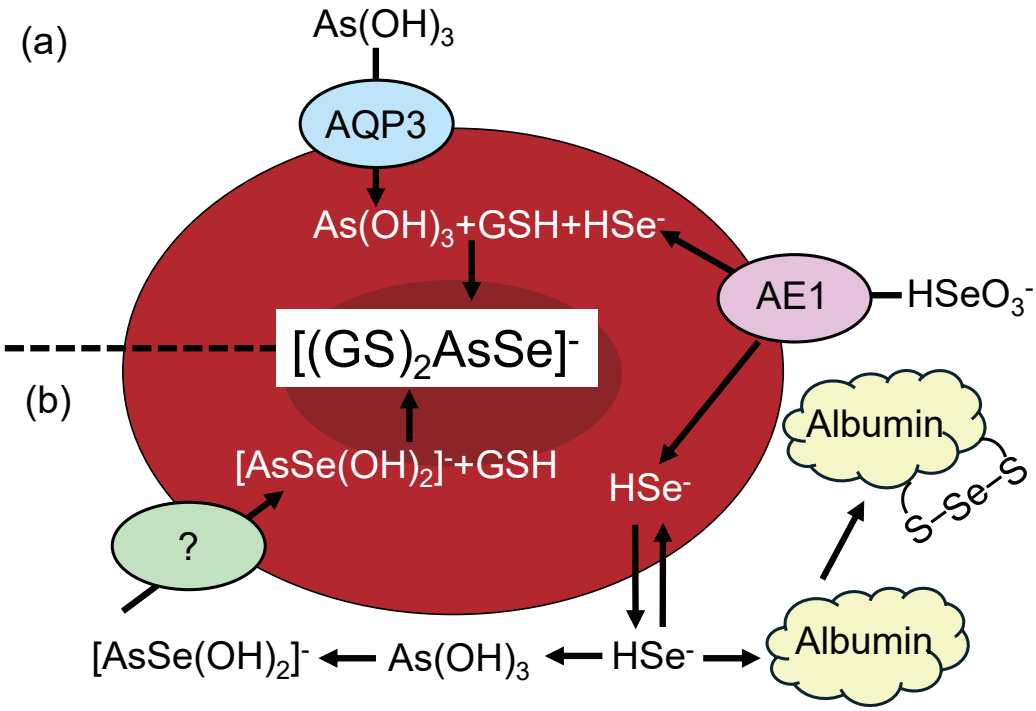

Fig. 5 **Two putative pathways for the enhancement of iAsIII and iSeIV uptake in erythrocytes when these two metalloids are doped simultaneously.** (a) proposed by Kaur G et al. [16] and (b) proposed in the present study.

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Author's contribution

Conceptualization: Y. K. and S. H.; methodology: Y. K. and S. H.; investigation: Y. K.; validation: Y. K. and S. H.; formal analysis: Y. K.; writing of the original draft: Y. K.; review and editing: S. H. and Y. K.; funding acquisition: Y. K.; supervision: S. H.

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Supplemental Information

Table S1 **Arsenic and selenium recovered from each sample.**
Selenite (iSeIV) (final 10 μM) and/or arsenite (iAsIII) (final 10 μM) were added to the erythrocyte suspension (final 10%) and then incubated at 37°C for up to 10 min. Values are presented as the mean ±SD (n = 3).

	(a) 10% erythrocytes	(b) 10% lysate	(c) 10% ghost	(d) supernatant	(e) Total (a+d)	(f) Total (b+c+d)
	Recovery of Se (% of the dose)					
iSeIV	69.4 ± 1.70	57.7 ± 3.40	2.45 ± 0.770	21.2 ± 0.90	90.6	81.4
iSeIV+iAsIII	83.7 ± 1.87	67.0 ± 3.09	4.10 ± 1.15	12.5 ± 0.392	96.2	102
	Recovery of As (% of the dose)					
iAsIII	14.4 ± 1.15	6.63 ± 0.190	0.456 ± 0.194	84.5 ± 1.88	98.9	91.6
iSeIV+iAsIII	84.8 ± 2.00	66.2 ± 3.37	3.48 ± 1.18	14.2 ± 0.629	99.0	83.9

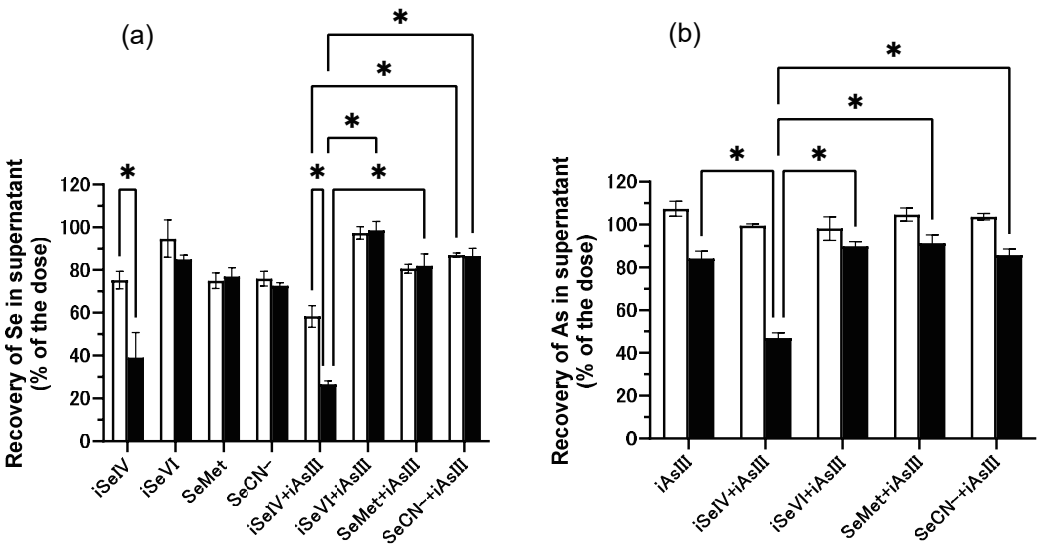

Fig. S1 **Accumulation of (a) Se and (b) As in the erythrocytes.**
Se compounds (final 10 μM), such as selenite (iSeIV), selenate (iSeVI), selenomethionine (SeMet), selenocyanate (SeCN-), and/or arsenite (iAsIII) (final 10 μM) were added to the erythrocyte suspension (final 10%) and then incubated at 37°C for up to 10 min. Erythrocytes and supernatants were separated by centrifugation (8,000 *g*, 10 s). Vertical bars indicate As or Se recovery (% of dose) from the supernatant. Open and closed bars represent before and after incubation, respectively. Values are presented as the mean ±SD (n = 3). Asterisks indicate significant differences between the samples. The experiment was performed in duplicate to ensure reproducibility.