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Selenomethionine Biotransformation and Incorporation into Proteins along a Simulated Terrestrial Food Chain

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Selenium is an essential trace element in vertebrates, but there is a narrow concentration range between dietary requirement and toxicity threshold. Although a great deal is known about the biochemistry of Se from a nutritional perspective, considerably less attention has been focused on the specific biochemistry of Se as an environmental toxicant. Recent advances in hyphenated analytical techniques have provided the capability of quantifying specific chemical forms of Se in biological tissues as well as the distribution of Se among macromolecules. We applied liquid chromatography coupled to inductively coupled plasma mass spectrometry to investigate biotransformations of selenomethionine along a simulated terrestrial food chain consisting of selenomethionine exposed crickets (*Acheta domesticus*) fed to western fence lizards (*Sceloporus occidentalis*). Evidence was obtained for selenomethionine biotransformation as well as for sex-specific differences in the metabolism of Se compounds and their subsequent incorporation into proteins in the lizard. The results demonstrate the complexities involved in trophic transfer of Se due to the potential for extensive biotransformation and the species- and even sex-specific nature of these biotransformations.

Introduction

Selenium is an essential trace element in vertebrates at sub-mg kg^{-1} dry mass dietary concentrations, but it can become a teratogen when dietary concentrations exceed a few mg kg^{-1} (*1*−*5*). Although a great deal is known about the biochemistry of Se from a nutritional perspective (summarized in Sunde et al., ref *5*), considerably less attention has been focused on the specific biochemistry of Se as an environmental toxicant. The vast majority of environmental studies have focused on determining total Se concentrations in biological tissues and environmental samples (*2*). A recent study investigating the speciation of Se in a contaminated environment suggested that selenomethionine ([Se]met), particularly in proteinaceous forms, may play an important role in food chain accumulation and subsequent toxicity of Se (*6*). This suggests that chemical speciation of Se plays an important role in determining its fate and effects in the environment.

Selenium has complex biogeochemistry and occurs in chemical forms that are analogous to forms of sulfur (S). Chief among these are elemental selenium (Se^0) selenide (Se^{-2}), selenite (SeO_3^-), and selenate (SeO_4^-) as well as methylated forms $Se_x(CH_3)_x$. Selenate and selenite can be taken up by plants and converted to organic forms (*2*, *6*−*10*). These organic forms are usually analogues to S-containing biomolecules, especially amino acids. This occurs through either nonspecific isosteric substitution for S in amino acids ([Se] cysteine or [Se]met) (*11*), or through co-translational conjugation of selenophosphate ($SePO_3^-$) to serine mediated by selenocysteine (sec) transfer RNA ($tRNA^{sec}$) and sec synthase (SELA) (*6*). In the latter case, sec is incorporated into genetically encoded selenoproteins (i.e., those proteins whose encoding DNA sequences have a UGA codon and a sec insertion sequence) (*12*). In addition, some other metabolites, such as seleno-sugars, are known to occur (*13*, *14*). Biotransformation and chemical speciation of both organic and inorganic contaminants is widely recognized as playing an important role in determining their fate and effects in the environment. Given the richness of biochemical pathways through which Se may be metabolized, it is important to understand what Se biotransformations may occur in organisms and how these transformations relate to food chain bioavailability, nutrition, and toxicity.

Recent advances in hyphenated analytical techniques have provided the capability of quantifying specific chemical forms of Se in biological tissues as well as the distribution of Se among macromolecules (*15*). Specific low molecular weight chemical forms of Se present in tissues are typically determined using ion chromatography (IC) with inductively coupled plasma mass spectrometry (ICP-MS) as an element-specific detector or by electrospray ionization mass spectrometry (ESI-MS) (*7*, *13*, *16*, *17*). Analysis by IC-ICP-MS can also be performed following enzymatic digestion of the tissue with broad spectrum proteases, enabling quantification of the seleno−amino acid composition of proteins. The sensitivity of ICP-MS as a Se detector has also been greatly improved since the introduction of the dynamic reaction cell (DRC) (*18*). The ICP-MS can also be used to detect high molecular weight Se-containing compounds, such as proteins, following size exclusion chromatography (SEC) (*19*−*21*) or laser ablation of polyacrylamide gels following separation by electrophoresis (*22*).

We applied SEC-ICP-DRC-MS and IC-ICP-DRC-MS to investigate biotransformations of [Se]met along a simulated terrestrial food chain consisting of [Se]met exposed domestic crickets (*Acheta domesticus*) fed to western fence lizards (*Sceloporus occidentalis*). A previous study we conducted showed that Se in *S. occidentalis* eggs and follicles was primarily present in proteinaceous forms and was distributed among these proteins according to their S content (*23*). Therefore, an additional objective was to determine if Se in liver tissue from male and female lizards as well as male gonadal tissue was distributed strictly according to S content and to determine if accumulated Se was present in these tissues in proteinaceous versus low molecular weight forms. Another previous study of western fence lizards fed crickets reared on a [Se]met-enriched diet showed that although male and female lizards accumulated similar whole-body concentrations of Se, the partitioning of the body burden among

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liver, gonad, and remaining carcass, as well as the resulting concentrations, differed between sexes (*24*). Therefore, our final goal was to determine if differences in molecular distribution and speciation of Se could help to provide an explanation for sex related differences in partitioning of Se among organ systems. We accomplished these objectives by examining the distribution of Se-containing biomolecules in whole crickets reared on [Se]met-enriched diets as well as on gonadal and liver tissues from male and female lizards reared on the [Se]met exposed crickets.

Experimental Section

Laboratory Exposures and Tissue Sampling. The analyses described here were performed on tissues archived at −70 °C from a study investigating accumulation of Se in western fence lizards exposed to [Se]met via a simulated terrestrial food chain as described elsewhere (*24*). Briefly, Se was first introduced into the live prey of lizards, the domestic cricket, by providing prey with Se-enriched diets. Diets were formulated by grinding Iams Chunks dog food into a fine powder, adding an appropriate amount of seleno-D,L-methionine ([Se]met; Sigma, St. Louis, MO; Se treatments) or control amino acid solution (D,L-methionine; controls) to the food matrix, and drying the food at 40 °C overnight. Selenomethionine was chosen over other chemical forms of Se because it is believed to be one of the primary Se species trophically transferred from prey to vertebrate predators and it is more toxic in the diet than inorganic forms of Se (*6*, *25*) The resulting whole-body Se concentrations for crickets ranged from 14.8 to 16.0 μg g^{-1}. Juvenile lizards received rations of crickets equaling 30% of their body mass per week. After 98 d on their respective diets, lizards had reached reproductive maturity. All lizards were euthanized and an egg or follicle, depending on which was present, was removed, flash frozen in liquid nitrogen, and stored at −70 °C. Subsamples of liver and testicular tissue (100−200 mg) were removed, flash frozen at the time of dissection, and stored under the same conditions. Lizards accumulated an average of 14.0 ± 0.22 μg g^{-1} (mean ± standard error of the mean) dry mass in ovaries and 10.6 ± 0.24 μg g^{-1} in testes. Liver tissue concentrations were 11.3 ± 0.90 and 12.6 ± 0.63 μg g^{-1} in female and male lizards, respectively. Concentrations in the remaining carcasses were 9.3 ± 0.26 and 10.6 ± 0.29 μg g^{-1} in female and male lizards, respectively.

Enzymatic Digestion and Anion Exchange Chromatography. Proteins present in tissue samples were enzymatically hydrolyzed with a broad spectrum protease (Pronase E, *Streptomyces griseus*, Sigma, St. Louis, MO) to yield individual amino acids. The samples were digested with 10% w/w protease at 37 °C in 100 mM, pH 7.5 phosphate buffer for 24 h followed by a second addition of 10% w/w protease and incubation for an additional 24 h (n = 4 for each tissue/sample type per treatment). This procedure was shown to quantitatively release Se species from biological tissues (*26*). Anion-exchange chromatography was performed with a PRP-X100 anion exchange column (Hamilton, Reno NV) using the method of Stadlober et al. (*7*). The mobile phase (10 mM, citrate buffer, pH 5.0, with 2% methanol) was pumped through the column at a rate of 1.5 mL min^{-1}. The LC system consisted of an inert autosampler (AS3500; Thermo Electron, Waltham, MA) and an inert pump (GP40; Dionex, Sunnyvale, CA). Standards were prepared by serial dilution of selenocystine (sec_2), seleno-D,L-methionine [Se]met, sodium selenate and sodium selenite (Sigma, St. Louis, MO). Selenocystine, which is a dimer of 2 sec moieties joined through a diselenide bond, is used instead of sec as a standard, because sec (which is not commercially available) rapidly oxidizes under aerobic conditions to form sec_2. Cys-sec dimers do not readily form because of the difference in redox potential between S and Se (*11*). Since it is the Se atom that is being quantified, there is no need to differentiate between the two species. Therefore, any sec present in the biological samples was quantified as sec_2.

The eluate from the IC column was introduced into an ICP-DRC-MS (Perkin-Elmer-Sciex Elan DRC plus, Shelton, CT) using a concentric nebulizer and a Peltier-cooled cyclonic spray chamber (Elemental Scientific, Omaha, NE). The DRC was pressurized with 5% hydrogen (H) in argon (Ar) at a flow rate of 0.4 mL min^{-1}. The low mass cutoff parameter (RPq) was set at 0.45. Use of the DRC suppressed the signal from $^{40}Ar^{40}Ar$ ions allowing for sensitive quantification of ^{80}Se. The element-specific chromatograms were collected in data-only mode at m/z 80 for Se in peak hopping mode with a dwell time of 100 ms per reading. The number of readings was adjusted so the replicate measurement time spanned the entire chromatographic separation.

Protein Extraction and Size Exclusion Chromatography. Tissue samples were homogenized in four volumes of ice-cold 50 mM Tris (pH 7.2) containing 0.1% Triton X-100 in pre-chilled glass micro-tissue grinders (n = 4 follicles or testes per treatment, for liver samples n = 3 composite samples consisting of tissue samples from four individuals where one composite sample was from males, one from females, and one combined male and female from each treatment). The homogenates were then centrifuged at 1200*g* for 20 min at 4 °C. The supernatants were decanted and analyzed without further preparation. The entire process was carried out either on ice or at 4 °C, and the supernatants were immediately injected onto the SEC column to avoid proteolysis. A protease inhibitor cocktail was not used as they may complicate the SEC chromatographs. The pellets were then lyophilized and along with small aliquots of the supernatant were digested in HNO_3 using a MARS 5 microwave digestion system (CEM, Mathews, NC). The digests were analyzed by ICP-DRC-MS to determine how much Se was recovered by the extraction process. Recovery of Se from follicles and eggs from the Se-exposed group was 20%, which is in the range of what is expected in aqueous extractions and also reflects the low solubility of yolk platelets (*10*, *11*). Recovery of Se from liver and testicular tissue averaged 50 and 53%, respectively.

Proteins in the supernatants were separated on a Superdex 10/30 200 HR SEC column (Amersham Biosciences, Piscataway, NJ) which has an effective size separation range of 10−600 kDa and a bed volume of 24 mL. The mobile phase was 100 mM Tris (pH 7.2) and flow rate was held constant at 0.5 mL min^{-1}. Absorbance was quantified at 210 nm using an inline ultraviolet (UV) absorbance detector (Alltech model 460, Deerfield, IL) to monitor the elution of biomolecules. The SEC column, which separates based on hydrodynamic radius, was calibrated from 12.4 kDa to 669 kDa using a molecular marker kit for SEC (Sigma, St. Louis, MO). This kit contained a number of metalloproteins which were detected by ICP-MS. The excluded and total volumes of the column (V_0 and V_t, respectively) were determined using blue dextran (2000 kDa) and acetone (58 Da) which were detected by UV absorbance. The eluate was introduced into the ICP-MS using a cooled cyclonic spray chamber and concentric nebulizer as described above. The ICP-DRC-MS was used under the conditions stated above. For SEC chromatograms, data were also collected at m/z 34 for S. The DRC conditions used to suppress $^{40}Ar^{40}Ar$ ions, in combination with the Peltier-cooled cyclonic spray chamber, also effectively reduced the background from $^{16}O^{18}O$ ions allowing for quantification of S. Sulfur intensity was recorded to determine if Se distribution reflected the normal distribution of S in proteins.

Results and Discussion

Biotransformation of Selenomethionine. As expected, Se in the cricket feed was almost entirely [Se]met (Figure 1).

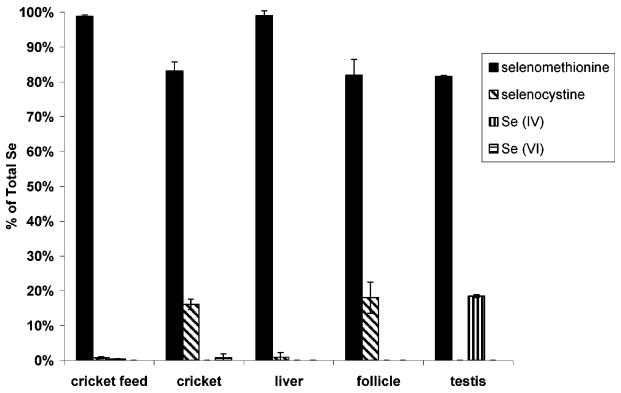

FIGURE 1. Selenium speciation in enzymatic hydrosylates from organisms in a simulated terrestrial food chain consisting of cricket feed, crickets (*Acheta domesticus*), and western fence lizards (*Sceloporus occidentalis*). Crickets were enriched in selenium by spiking their diet with 30 ug g $^{-1}$ seleno-D,L-methionine. Liver tissue, ovarian follicles, and testes were analyzed for lizards, and whole bodies were analyzed for crickets.

Minute traces of sec_2 (1% of total Se) and selenite (0.4% of total Se) were also present. A considerable fraction (16%) of the Se in crickets was in the sec_2 form. Selenium compounds in control crickets and feed were not detectable using this method. This suggests that crickets have significant capacity to metabolize [Se]met. The pathway for metabolism of [Se]-met involves syntheses of adenosyl [Se]met followed by transmethylation to adenosyl selenohomocysteine (*27*). Adenosyl selenohomocysteine is a substrate for synthesis of selenocystathionine by cystathionine β-synthase and subsequent lysis by cystathionine γ-lyase yields sec (*5*). These pathways leading to sec primarily result from isosteric substitution of [Se]met for methionine into S pathways (*5*), but they also allow organisms to synthesize specific selenoproteins using [Se]met as a starting material, which is the major Se species present in non-accumulator plants(10,28), rather than through reduction of selenate or selenite (*5*). It is probably inappropriate to consider conversion of [Se]met to sec as part of a detoxification pathway, as [Se]met would be more readily detoxified in animals through transamination to α-keto-8-[Se]methiolbutyrate, conversion to methane selenol as an intermediate and subsequent transmethylation to demethylselenide or the trimethylselenonium ion. Direct methylation of [Se]met and subsequent lysis of methylated Se may also possible (*29*, *30*). The aforementioned intermediates in Se metabolism are usually present in very low concentrations in organisms, except in plants that hyperaccumulate Se, and they are typically not detected by IC-ICP-MS nor is dimethylselenide (*7*, *10*). The intermediate selenocystathionine and the trimethylselenonium ion also have retention times that are very similar to sec_2 at pH 5.0 making them difficult to separate (*7*); however, the chromatograms from SEC-ICP-MS of ovarian follicles and eggs confirms the identity of sec_2 in these chromatograms as there was no evidence of such low molecular weight compounds (*23*).

The liver tissue contained predominately (99%) [Se]met, with the remaining fraction present as sec_2. The predominance of [Se]met over sec in liver tissue may have resulted from preferential accumulation in the liver of [Se]met over sec from the crickets or metabolism and elimination of sec as methylated species. Animals cannot synthesize [Se]met due to a lack of pathways for biosynthesis of met, which is ultimately obtained from plants. Therefore, it is highly unlikely that accumulated sec was converted to [Se]met. Once again similar Se compounds were not detected in control liver tissue.

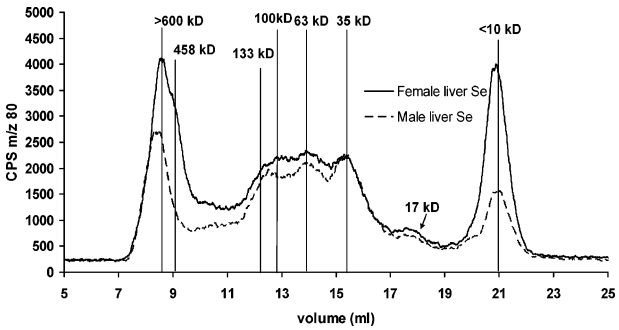

FIGURE 2. Size exclusion chromatographs of selenium content of proteins extracted from pooled male and pooled female liver tissue from the western fence lizard (*Sceloporus occidentalis*) exposed to Se enriched crickets. Selenium concentrations were obtained by monitoring *m/z* 80 with an inductively coupled plasma dynamic reaction cell mass spectrometer. Female liver is shown with a dotted line and male liver is shown with a solid line.

Selenium speciation in ovarian follicles and testes was quite different. Ovarian follicles contained an average of 18% sec_2, which is similar to the sec_2 content of the crickets (Figure 1). Testes accumulated the same fractional amount as selenite. Control follicles and testes did not contain detectable quantities of these compounds. It is possible that sec_2 may be converted to [Se]alanine by cys dioxygenase and subsequently selenite by desulfinase, analogous to S metabolism, although it is thought to be more common for Se^{2-} to be lysed directly from sec lyase (*31*). It is also possible that selenite could form on oxidation of sec during analysis; however, given that selenite was not observed in the standards or in other tissues we find this unlikely. We cannot rule out nonenzymatic oxidation of sec or [Se]met in vivo. The presence of selenite in the testes also confirms the presence of sec in lizard tissues, as selenite can only be derived from selenate or sec (*5*). These data suggest that there may be unique Se metabolism in lizard testicular tissue compared to other tissue types. It has been demonstrated that rat testes specifically accumulate Se and that Se content is generally maintained at high levels at the expense of other tissues (*32*). Involvement of Se in the testis is critical for spermatogenesis, in part because Se is contained in the sperm mitochondira capsule protein and in phospholipid glutathione peroxidase (GPX-4) (*33*). Little is known concerning the specific uptake or metabolism of Se compounds in the testes; however, it is possible that metabolic activity specific to the testes results in efficient conversion of sec to selenite. Regardless, the presence of selenite rather than sec in the testes is a key difference between accumulation of Se in male and female gonads. Although only a few samples were analyzed for each tissue type/species, the relative proportions of the various Se species were very consistent.

Molecular Weight Distribution of Selenium-Containing Proteins. A wide band of Se-containing proteins was present in both male and female liver tissues that ranged in mass from 33 kDa to 135 kDa (Figure 2). This band was sometimes partially resolved into up to four peaks with estimated molecular weights of 35, 63, 100, and 133 kDa. The 100 kD peak seemed to be slightly more abundant in female tissue. A smaller peak, present in both male and female liver tissue also occurred at 17 kDa. These peaks were of comparable magnitude per unit mass of tissue in both male and female lizards. Large peaks that occurred in the V_0 of the column also occurred in both male and female liver tissue. This peak represents particles that are larger than 600 kDa and are most likely protein aggregates. A shoulder that had an estimated size of 458 kDa was sometimes present on the peak for the V_0 in female liver tissues. This was the only obvious difference between the distribution of Se in male

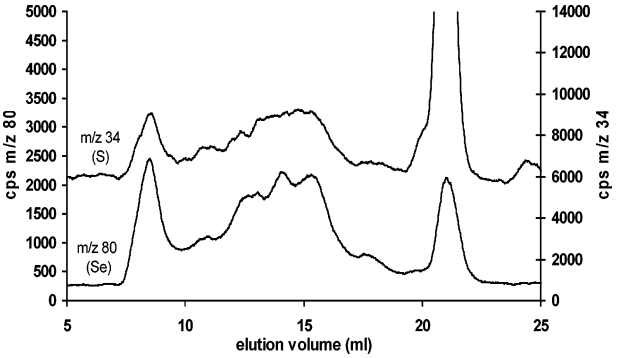

FIGURE 3. Size exclusion chromatographs of selenium and sulfur content of proteins extracted from pooled male and female liver tissue from the western fence lizard (*Sceloporus occidentalis*) exposed to Se enriched crickets. Selenium concentrations were quantified by monitoring *m/z* 80 and S concentrations were quantified by monitoring *m/z* 34 with an ICP-DRC-MS.

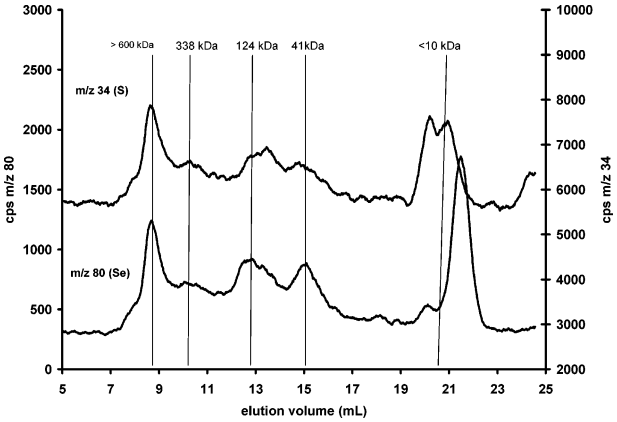

FIGURE 4. Size exclusion chromatographs of selenium and sulfur content of proteins extracted from testicular tissue from the western fence lizard (*Sceloporus occidentalis*) exposed to Se enriched crickets. Selenium concentrations were quantified by monitoring *m/z* 80 and S concentrations were quantified by monitoring *m/z* 34 with an ICP-DRC-MS.

and female liver proteins. A large peak that eluted near the V_t of the column was also present in liver tissue from both sexes. This peak corresponded to low molecular weight (<10 kDa) Se-containing compounds, suggesting it corresponds to small Se-substituted peptides such as glutathione (GSH) which has a similar retention time; however, this result should be confirmed by ESI-MS. In the S chromatogram this peak was extremely large relative to the higher molecular weight S-containing peaks, while for Se the low molecular weight peak was of the same order of magnitude as the high molecular weight Se-containing peaks. Large amounts of GSH present in the low molecular weight S peak could explain this, since the S containing moiety in glutathione is cys, and relatively little sec was present in the liver tissue. The distribution of Se-containing compounds in liver corresponded fairly well to the distribution of S containing compounds (Figure 3), which is consistent with isosteric substitution of [Se]met for met in peptides. Some small differences are apparent between the S and Se chromatograms. For example, the band of Se proteins with molecular weights ranging from 35 to 166 kDa seems to consist of fewer, better resolved peaks than in the corresponding band of S proteins. It is logical that there should be some differences given that the liver tissue primarily accumulated [Se]met, whereas S-containing proteins could have either cys or met residues.

The ovarian follicles and eggs contained three distinct Se-containing proteins (chromatogram not shown). As described elsewhere (*23*), the largest protein from the eggs is lipovitellin and is a homodimer with a subunit molecular weight of 111 kDa. The other two proteins, which were present in both follicles and eggs, are of unknown identity. One is a 75 kDa protein is a monomer and may be livetin, a major egg protein similar to serum albumin, while the 45 kDa protein is a heterodimer. These proteins may play an important role in maternal transfer of Se (*23*). The SEC chromatograms for Se and S corresponded to each other very closely in ovarian follicles and eggs, and almost all of the Se was incorporated into proteins. This result confirms the identity of sec or sec_2 in the enzymatic hydrosylate because the compounds that may be expected to elute near this retention time by IC-ICP-MS ([Se]cystathionine and the trimethylselenonium ion) would have eluted in the low molecular weight fraction near the V_t of the column. It is reasonable to expect that ovarian tissue would have a greater correspondence between the molecular weight distribution of S and Se than other tissues, given that it was the only lizard tissue type that contained appreciable amounts of sec_2 and [Se]met allowing for substitution in place of both S-containing amino acids.

Testicular tissue contained three major bands of Se-containing proteins centered at 338, 124, and 41 kDa as well as a large low molecular weight (<10 kDa) peak (Figure 4). The testicular tissue, like the liver tissue, also contained a peak near the V_0 of the column, which most likely corresponded to protein aggregates. Correspondence between the Se- and S-containing chromatograms was not as good for testicular tissue as it was for the other tissues, primarily due to differences in the low molecular weight peaks. The chromatogram for S also had a double peak just before the V_t, while the Se chromatogram did not have these peaks. The most striking feature of the SEC chromatograph for Se was a very large peak eluting well after the V_t of the column. This peak most likely corresponds to selenite, as it had the same retention time as a selenite standard and was not present in the other tissues, which did not contain selenite. Further, other low molecular weight species (met, sec_2, selenate) had different retention times. Very low molecular weight compounds can have electrostatic interactions with the SEC medium causing charged species, such as selenite, to be sorbed and elute after the V_t. Control tissues contained such small quantities of Se incorporated into proteins that they were not detected using this method. Although only a small number of samples were analyzed, the SEC chromatograms were qualitatively consistent among individual or pooled samples for each tissue type.

We have shown that terrestrial organisms may play important roles in biotransformation of dietary [Se]met. The basal resource in our experimental food chain was spiked with [Se]met, but a considerable fraction of Se was present as sec after the first trophic transfer. This has important implications, because it increases the potential for sec substitution into proteins. Because Se has different redox properties than S, the functionality of these residues in structural proteins, enzymes, and small peptides such as GSH, may be compromised. For example, substitution of sec in place of cys may disrupt sulfur bridges because of the different redox potentials at which diselenide and disulfide bridges form. Also, met on the surface of proteins can have important antioxidant properties which would be lost as result of the readiness of [Se]met to oxidize. Methionine also serves as the methyl donor in several biochemical reactions which may be altered as a result of substitution by [Se]met (*11*).

We have also demonstrated that the molecular weight distribution of Se proteins extracted from various tissues in lizards is similar or nearly identical to the molecular weight

distribution of S. The biggest differences seen in the molecular weight distribution of Se and S in biomolecules were generally in the low molecular weight compounds, most likely due to differences in metabolism of inorganic S and Se species caused by differences in their redox properties. For example, Se has a higher reducing potential and may, therefore, be more readily oxidized than S. Differences and similarities in tissue concentrations of Se between sexes are also readily explained by differences in low molecular weight Se species present and differences in the molecular weight distribution of Se in proteins. Given that Se nonspecifically substitutes for S in proteins, accounting for most of the accumulated Se, it is ultimately sex-specific tissue differences in gene expression that govern these differences in bioaccumulation between the sexes. This finding also has implications for differences in Se bioaccumulation among species. Species differences in cys and met content of specific proteins could have important implications for the propensity of a given species to bioaccumulate Se/the adverse toxicological effects that are expressed. This suggests that genetic differences and differences in gene expression among and within species may play a role in determining patterns of bioaccumulation and distribution of Se among organ systems. This may help to explain the variability, both within and among species, in sensitivity to Se exposure (*35*). Interspecies variability in Se bioaccumulation can also have important implications for transport of Se through food webs and subsequent adverse effects in receptor species (*34*). Our findings highlight the importance of considering the biochemistry and molecular biology of Se to understand the complex mechanisms by which this element is transported through food webs and manifests adverse effects in wildlife.

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