Metabolism of selenite to selenosugar and trimethylselenonium in vivo: tissue dependency and requirement for S-adenosylmethionine-dependent methylation☆

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Abstract

Impaired S-adenosylmethionine (SAM)-dependent transmethylation and methylation capacity feature in diseases related to obesity or aging, and selenium (Se) metabolism is altered in these states. We tested the hypothesis that SAM metabolism is required for methylation and excretion of Se in a rat model. Four hours after selenite and periodate-oxidized adenosine (POA; an inhibitor of SAM metabolism) were administered, circulating markers of single-carbon status were unchanged, except for decreased circulating phosphatidylcholine (P=0.05). In contrast, liver and kidney SAM and S-adenosylhomocysteine were elevated (P<0.05 for all). Concentrations of total Se were significantly elevated in both liver (P=0.001) and kidney (P=0.01), however the degree of accumulation in liver was significantly greater than that of kidney (P=0.05). Red blood cell Se levels were decreased (P=0.01). Trimethylselenonium levels were decreased in liver and kidney (P=0.001 for both tissues) and Se-methyl-N-acetylselenohexosamine selenosugar was decreased in liver (P=0.001). Urinary output of both trimethylselenonium (P=0.001) and selenosugar (P=0.01) was decreased as well. Trimethylselenonium production is more inhibited by POA than is selenosugar production (P=0.05). This work indicates that low molecular weight Se metabolism requires SAM-dependent methylation, and disrupting the conversion of SAM to S-adenosylhomocysteine prevents conversion of selenite and intermediate metabolites to final excretory forms, suggesting implications for selenium supplementation under conditions where transmethylation is suboptimal, such as in the case of obese or aging individuals.

Keywords: Selenium; Selenite; Selenosugar; Trimethylselenonium; Methylation; Liver; S-adenosylhomocysteine; S-adenosylmethionine; Cancer

1. Introduction

Dietary selenium (Se) undergoes non-specific incorporation of selenomethionine (SeMET) in place of methionine during general protein synthesis [1,2] as well as co-translational incorporation into selenoproteins as selenocysteine [3,4]; selenoproteins fulfill diverse, essential biological roles [5–8], and play a role in the anti-cancer activity of Se [9]. Food forms of Se, are also metabolized by reduction and methylation [10,11] to various low molecular weight species including trimethylselenonium ion (TMSe⁺), Se-methyl-N-acetylselenohexosamine (Se-SUG) and the seleno-amino acids Se-methylselenocysteine (MeSeCys) and SeMET. The oxidation state of low molecular weight Se determines its potential for reacting with regulatory cysteine residues in signaling proteins [12], while methylation state imparts hydrophobicity to affect membrane accessibility and prevent further redox cycling [13]. Excess Se can be eliminated in urine after methylation-dependent metabolism to either TMSe⁺ [14,15] or a series of Se-SUG species which comprise the major urinary metabolites of Se in humans [16,17] (Fig. 1). Low molecular weight Se redox cycles to generate reactive oxygen species [18], induces apoptosis [19], covalently modifies thiols [20], alters cell cycle progression [21,22] and protects against the toxic effects of certain chemotherapeutics through maintenance of circadian rhythm [23]. The anticancer activity of Se [24–26] at supranutritional levels appears to be due to the action of low molecular weight, methylated Se-metabolites [27,28]. Individuals vary in their capacity to transform ingested Se to methylated species [29,30]. Genetic, sex and physiological factors, account for differences in selenoprotein expression [31–34], but since little is known about the factors influencing inter-individual differences in metabolism of low molecular weight Se it is of interest to elucidate these determinants in vivo.

Portions of the whole-body Se pool existing in protein and low molecular weight form are metabolically exchangeable with selenite experimentally administered by injection in both human subjects and rat models [35]. This metabolic pool of Se is termed the “selenite-exchangeable pool” [36–38]. The selenite-exchangeable pool is correlated with total body Se in vivo [39,40], although it can be perturbed in vivo by nutritional supplementation with the reducing agent ascorbic acid [41]. We tested the hypothesis that impairment of S-adenosylmethionine (SAM) dependent methylation decreases metabolism of the selenite-exchangeable pool to low molecular

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Fig. 1. The transmethylation cycle includes SAH, a potent feedback inhibitor of SAM-dependent methyltransferases and homocysteine, a marker of methylation capacity. Dietary forms of Se include SeMET, selenocysteine (SeCys), selenite and MeSeCys. Whereas SeMET is converted to other non-protein Se after going through the transmethylation cycle, SeCys/ selenite and MeSeCys are converted directly to hydrogen selenide (H₂Se) and methylselenol (CH₃SeH) respectively. From the central metabolite H₂Se, excretory forms of methylated non-protein Se, trimethylselenonium ion ([CH₃]₃Se⁺) and selenosugar (CH₃-SeSUG), are produced with successive inputs of SAM and the actions of methyltransferases. CH₃-SeSUG only requires the input of one SAM, whereas the formation of [CH₃]₃Se⁺ requires three SAM.

Fig. 2. POA treatment increased levels of SAM (A,C) and SAH (B,D) in both liver (A,B) and kidney (C,D). The p values for individual comparisons are indicated in the figure, with significance set to P < .05.

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weight forms by treating rats with periodate oxidized adenosine (POA), a reversible inhibitor of S-adenosylhomocysteine (SAH) hydrolase (AHY; EC 3.3.1.1) that induces accumulation of SAH [42,43]. SAH is an enzymatic product of SAM dependent methyltransferases and a potent feedback inhibitor of these enzymes (Fig. 1). POA treatment has previously been shown to reduce the excretion of dimethylselenide in the rat [44–46]; however, those studies took place prior to the discovery of Se-SUG as a predominate excretory product of Se metabolism. Here we report the effects of the inhibition of SAM-dependent methylation capacity on the production and excretion of low molecular weight Se species.

2. Methods and materials

2.1. Animal care and diet

Male, weanling Sprague-Dawley rats were purchased from Harlan Labs (Madison, WI, USA), weighed upon arrival, and assigned to each dietary group (N=12–13/group) with no significant differences in initial weight among groups. Rats were provided free access to demineralized water and a purified diet in a room with controlled temperature and light. The basal amino acid-based diet was AIN-93G [47]; by analysis it contained 0.49 ± 0.01 ppm Se, a level that is considered nutritionally adequate and is sufficient to maximally express selenoproteins. Rats were fed this diet for 14 days after which they were randomized into two groups (selenite alone, n=12; selenite + POA, n=13). Each group was administered a single dose of 10 μmol/kg selenite, additionally one of the groups was co-administered a single dose of 7.5 μmol/kg POA by intraperitoneal injection (compounds dissolved in sterile saline). Four hours after dosing, rats from both groups were anaesthetized by intraperitoneal injection of ketamine (58 mg/100 g rat weight) plus xylazine (8.4 mg/100 g rat weight) and euthanized by severing the diaphragm. Samples of blood, liver and kidney were obtained and snap frozen in liquid nitrogen prior to storage at -80°C until analysis. Urine samples were captured by bladder puncture and frozen at -80°C. This study was approved by the Animal Care Committee of the Grand Forks Human Nutrition Research Center, and the rats were maintained in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

2.2. Chemicals and materials

All reagents were analytical grade. POA, methylseleninic acid, sodium selenite, sodium selenate, SeMET, ammonium acetate and formic acid were from Sigma-Aldrich (Steinheim, Germany). Oxalic Acid was from BDH Chemicals ltd (Pool Dorset, England). Methanol was from VWR (VWR-Bie & Berntsen, Herlev, Denmark). K2SO4, NaOH and HNO3 were from Merck (Darmstadt, Germany); the nitric acid was purified by sub-boiling twice before use. The columns used for LMW Se speciation were from the following sources: Luna C18 100×2 mm; Phenomenex, Allerød, Denmark; Gemini C18 250×2 mm; Phenomenex, Allerød, Denmark; Hypersil BDS 250×2 mm; Thermo Scientific, Copenhagen, Denmark; C55A 250×4.6 mm; Dionex, Sunnyvale, CA, USA; AS-11-HC 250×2 mm; Dionex, Sunnyvale, CA, USA.

2.3. Enzyme and biochemical assays

Plasma glutathione peroxidase (GPX3); The activity of GPX3 (E.C. 1.11.1.9) was determined in plasma by the method of Paglia and Valentine [48] as modified by Lawrence and Burk [49]. Creatinine: urine creatinine was analyzed on an automated chemistry analyzer (Cobas Integra 400 Plus, Roche Diagnostic Systems, Sommerville, NJ, USA). Vitamin B12, folic acid and homocysteine were measured on an automated chemistry analyzer (Siemens Immunolite 1000, Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA). Phosphatidylcholine: Plasma phosphatidylcholine was measured using a kit from Cayman Chemical (Ann Arbor, MI, USA).

2.4. S-adenosylmethionine and SAH measurement

Tissue samples (0.2–0.4 g) were powdered in liquid nitrogen, weighed and homogenized in 0.75 M perchloric acid on ice with a Potter-Elvejem tissue grinder. Samples were clarified by centrifugation, and the supernatant snap frozen in liquid nitrogen before storage at -80°C until analysis. Urine samples were captured by bladder puncture and frozen at -80°C. This study was approved by the Animal Care Committee of the Grand Forks Human Nutrition Research Center, and the rats were maintained in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

2.5. Total selenium analysis

Selenium was determined by automated electrothermal atomic absorption spectrophotometry using a reduced palladium matrix modifier [51]. Certified Standards were used (Alfa Aesar (Ward Hill, MA, USA), Perkin Elmer (Waltham, MA, USA).

Fig. 3. Blood markers of single-carbon status are minimally impacted by POA treatment, with only plasma phosphatidylcholine significantly decreased (A) while plasma homocysteine (B) and serum vitamin B-12 (C) and folic acid (D) remained unchanged. The p values for individual comparisons are indicated in the figure, with significance set to P<.05.
USA) and CPI (Santa Rosa, CA, USA) for preparation of a calibration set. Matrix effects for plasma and urine were evaluated using certified reference material plasma and urine standards (National Institute of Standards and Technology (Gaithersburg, MD, USA), Seronorm (Billingstad, Norway) and Utak (Munich, Germany)).

2.6. Sample preparation for Se speciation

Samples were stored at −80°C until pretreatment. A Heidolph homogenizer was used where indicated. Urine: The urine samples were thawed, diluted 1:1 with Milli Q water and clarified by centrifugation and the supernatant transferred to autosampler vials. Plasma: 100 µl aliquots of rat plasma were added to 100 µl 0.1% formic acid in methanol, mixed and left at 4°C for 16 h to precipitate proteins. Following precipitation, the protein was pelleted by centrifugation and the supernatants evaporated under nitrogen flux. The samples were reconstituted in deionized water and clarified by centrifugation prior to analysis. Red blood cells (RBC), liver and kidney: RBC were homogenized in 50% methanol, followed by centrifugation and transfer of supernatants. Extraction was repeated twice, with supernatants pooled and evaporated before reconstitution in deionized water. Centrifugation again clarified reconstituted solutions prior to analysis. Results from kidney and liver were transformed from wet to dry weight data by using the average wet to dry weight ratio for each tissue.

2.7. Analysis of low molecular weight Se

HPLC: Chromatography was carried out on Agilent 1100 and 1200 series systems (Agilent, Waldbronn, Germany). Columns, mobile phases and flow rates used in the specific setups are listed in Supplementary information online (Table S1). ICP-MS: ICP-MS instruments were the Elan DRCe and Elan 6000 (Perkin Elmer SCIEX, Norwalk, CT, USA), using temperature controlled cyclonic spray chambers and microconcentric nebulizers. Nebulizer gas flow, lens voltage and RF power were optimized daily on a 100 ppb standard in mobile phase. The DRCe was used in DRC mode with methane as reaction gas at a flow of 0.55 L/min. Masses measured were: 78, 80 and 82 for the DRCe and 77, 78 and 82 for the Elan 6000. Peaks in chromatograms were identified as methylated Se-metabolites or methylated selenoamino acids by co-elution with known standards, and standard addition added to samples.

2.8. mRNA expression analysis (qPCR)

Total cellular RNA was manually prepared with Trizol (Invitrogen; Grand Island, NY, USA), cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Carlsbad, CA, USA) and quantified with the TaqMan Fast Advanced Master Mix on the 7500 Fast rtPCR instrument from Applied Biosystems. Taqman Primer assays were as follows: SEPP1 — Rn00569905_m1; GPX1 Rn00577894_g1. Gene expression was normalized to eukaryotic 18S rRNA. qPCR was performed on the 7500 Fast RT-PCR system (Applied Biosystems).

2.9. Statistical analysis

The relative effect of POA treatment on accumulation of kidney vs liver Se or on Se-sugar vs. TMSe excretion was tested by log-transforming Se concentrations and using a 2×2 mixed-model analysis of variance (ANOVA) with rat as a random effect. Variance estimates were allowed to be unequal for the two organs and for the two Se forms. An a priori contrast was included to test whether the difference in log(Se) concentrations between POA-treated rats and controls was significantly different between the two organs or the two forms of Se. The Glimmix procedure in SAS V9.3 (SAS Institute, Inc, Cary, NC) was used for the analyses.

3. Results

3.1. Levels of SAM, SAH and biomarkers of single-carbon status

Levels of liver SAM (P<.01) and SAH (P<.05) were significantly increased by POA treatment (Fig. 2) relative to the control group receiving selenite alone. Kidney SAM (P<.01) and SAH (P<.01) were also increased by POA treatment. Plasma phosphatidylcholine was slightly, but significantly decreased by POA treatment (P<.01), but levels of other markers of single-carbon status including homocysteine, serum vitamin B12 and folic acid were not significantly altered by POA treatment (Fig. 3).

3.2. Total Se in metabolic and circulating pools

Plasma levels of total Se were unchanged by POA, but this treatment induced a decrease in RBC total Se (P<.01) and an increase in both kidney (P<.001) and liver (P<.001) total Se. The increase of total Se in liver induced by POA treatment (50% above control value) was significantly greater (P<.01) than the increase of total Se in kidney (15% above control value) (Fig. 4).

3.3. Selenoprotein mRNA expression

Relative levels of mRNA transcripts of selenoprotein P (SEPP1; P<.41) and GPX1 (P<.71) in liver were not significantly altered by POA treatment (data not shown). Additionally, activities of plasma and tissue GPX were not changed in response to POA. Control and POA treated GPX activities were, respectively: 3405.1±544.2 and 3176.5±879.9 nmol min⁻¹ mg⁻¹ protein (liver GPX1); 956.7±52.9 and 928.9±177.3 nmol min⁻¹ mg⁻¹ protein (kidney GPX1+GPX3); 100.4±13.5 and 102.2±13.9 nmol min⁻¹ mg⁻¹ protein (plasma GPX3).

Fig. 4. Plasma total Se (A) was unchanged by POA treatment, however RBC (B) had a slight albeit significant decrease in total Se. Both liver and kidney (C) had an increase in total Se, and the magnitude of total Se accumulation was significantly greater in liver than in kidney (C). The p values for individual comparisons are indicated in the figure, with significance set to P<.05.
3.4. Low-molecular-weight Se in circulating and metabolic pools

Integrations of all peaks in chromatograms of sample extracts from POA-treated and untreated rats were used to estimate the amount of total low molecular weight Se species present in tissues. Plasma and RBC levels of total non-protein Se were statistically unchanged by POA (Fig. 5); however, the treatment induced significant accumulation of low molecular weight Se in liver (P < .01) and decreased levels in kidney (P < .001).

3.5. Methylated Se-metabolites and selenoamino acids in circulating and metabolic pools

Peaks in chromatograms were identified as methylated Se-metabolites or methylated selenoamino acids by co-elution with known standards under two orthogonal sets of separation conditions (Supplementary Table S1), and by standard addition added to samples. While our analyses have been published and validated with independent detection methods [17,30], it should be noted that identification based on retention-time co-elution with authentic standards is tentative. Levels of TMSe⁺ were significantly decreased in RBC (40% of control, P < .001), liver (32% of control, P = .001) and kidney (62% of control, P < .001) (Table 1). Levels of Se-SUG were decreased in kidney (57% of control, P < .001), but unchanged in liver, plasma and RBC. Further, levels of methylated Se-containing amino acids increased in liver after POA treatment; SeMET was increased to 220% of control values (P = .001) while MeSeCys increased to 240% of control values (P < .001). Levels of methylated Se-containing amino acids were unchanged in kidney, plasma and RBC.

3.6. Urinary excretion of methylated Se-metabolites

POA treatment decreased total levels of low molecular weight Se species in urine by 50% below control value. This change could be accounted for by decreases in both Se-SUG (25% below control value; P = .014) and TMSe⁺ (82% below control value; P < .001) (Fig. 6). Levels of total Se in urine, including protein bound forms of Se with masses of less than ~30 kDa did not significantly differ between control (12.1 ± 6.8 mg Se/g creatinine) and POA treated (10.44 ± 6.1 mg Se/g creatinine) groups. The decrease in urinary excretion of TMSe⁺ was significantly greater relative to the decrease in excretion of Se-SUG.

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Fig. 5. Plasma (A) and RBC (B) low molecular weight Se was unchanged by POA treatment. In contrast, liver (C) experienced an increase in non-protein Se while kidney (D) levels of low molecular weight Se were significantly decreased. The P values for individual comparisons are indicated in the figure, with significance set to P < .05.
of Se-SUG ($P < .001$), similar to the effect POA treatment had on the relative decrease of these Se-metabolites in kidney.

4. Discussion

The methionine/homocysteine cycle shuttles single-carbons from 5-methyltetrahydrofolate, via methyl-B12, to production of SAM, a substrate for methyltransferases acting on low molecular weight Se. Approaches to investigating methylation of Se have included altering dietary levels of vitamin B12 [52,53] or methionine [54]. Alternatively, we used POA, an indirect inhibitor of SAM-dependent methylation [43,44] that alters Se metabolism [44–46]. We applied POA in a rat model that examines the acute effect of limiting methylation capacity on production of species from the selenite exchangeable pool [35–41]. Limitations of this study include the large amount of Se provided in the treatment, which is supraphysiological relative to what humans ingest, the potential for differential metabolism of nutrients delivered by ingestion versus intraperitoneal injection, and the tentative nature of identification of Se metabolites by retention time matching to pure standards rather than by mass spectrum. The high level of Se was employed in part on the desire to provide a bolus amount of Se sufficient to inundate potential routes of metabolism, and also to provide enough Se such that metabolic pathways having low affinity for selenite would have sufficient substrate to produce products. Injection was chosen as a route of administration in favor of feeding high dietary levels of Se, as we sought to avoid chronic induction of Se metabolizing enzymes by low affinity for selenite would have sufficient substrate to produce products. Injection was chosen as a route of administration in favor of feeding high dietary levels of Se, as we sought to avoid chronic induction of Se metabolizing enzymes which might alter the pattern of low molecular weight Se from that might otherwise occur in individuals naive to high-dose Se. Although the identification lacks the authenticity of molecular mass spectra, the use of Se isotope specific ICP-MS detection and previous validation of this methodology [17,30] provides some measure of confidence in our assignment of the methylated Se metabolites. We validated our model of acute disruption of SAM-dependent transmethylation by confirming that POA treatment increases levels of SAM and SAH in both liver and kidney without impacting systemic markers of single-carbon status.

Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>LMW species</th>
<th>POA</th>
<th>Control</th>
<th>t-stat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>TMSe+</td>
<td>262±58</td>
<td>829±112</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>MeSeCys</td>
<td>449±37</td>
<td>419±49</td>
<td>0.346</td>
</tr>
<tr>
<td></td>
<td>SeMET</td>
<td>21±2</td>
<td>21±2</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>Se-SUG</td>
<td>30±3</td>
<td>53±6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma</td>
<td>TMSe+</td>
<td>trace</td>
<td>trace</td>
<td>0.656</td>
</tr>
<tr>
<td></td>
<td>MeSeCys</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Se-SUG</td>
<td>trace</td>
<td>trace</td>
<td>0.877</td>
</tr>
<tr>
<td>RBCs</td>
<td>TMSe+</td>
<td>2±1</td>
<td>5±1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>MeSeCys</td>
<td>trace</td>
<td>trace</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td>Se-SUG</td>
<td>3±3</td>
<td>2±1</td>
<td>0.335</td>
</tr>
<tr>
<td>Liver</td>
<td>TMSe+</td>
<td>110±20</td>
<td>178±28</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>MeSeCys</td>
<td>175±41</td>
<td>72±14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Se-SUG</td>
<td>20±4</td>
<td>9±1</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values for liver and kidney are in ng Se/g tissue (dry weight) while values for plasma and RBCs are in ng Se/ml and ng Se/g, respectively, $n=12$; statistical significance was ascertained by ANOVA.

Fig. 6. Low molecular weight Se species were decreased in urine (A), among them being Se-SUG (B) and TMSe+ (C). The magnitude of decrease in TMSe+ was greater than the decrease observed for Se-SUG. The $p$ values for individual comparisons are indicated in the figure, with significance set to $P < .05$.

TMSe+ is largely a terminal Se metabolite, Se-SUG can be metabolically recycled to serve as substrates for production of selenoproteins [55], indicating that Se-SUG is hydrolyzed or oxidized in vivo to low molecular weight methylselenol directly or via intermediacy of the selenoprotein precursor hydrogen selenide, which is itself biologically interconvertible with methylselenol [56]. Additional evidence for the in vivo conversion of Se-SUG to methylselenol stems from the observation that rats consuming Se-SUG excrete TMSe+ in the urine, proposed to occur by metabolic transformation of the Se-SUG to methylselenol, possibly via intermediacy of hydrogen selenide [57]. Another instance of this reaction in biological systems is found in the capacity of Brassica rapa species to produce the glutathione selenosulfide adduct of methylselenol upon treatment with Se-SUG, an adduct likely formed by reaction of reduced glutathione with
redox congeners of methylselenol or hydrogen selenide [58]. Chemical precedent for the formation of methylselenol from Se-SUG observed in biological systems comes from the oxidative degradation of Se-SUG to monomethyl species that has been documented in chemical systems and as an artifact of sample collection [59]. Methylselenol has disparate biological activity; it is the putative Se species largely responsible for the anticancer properties of Se [27,28], but can also potentially be the agent mediating the pro-diabetogenic effects of Se [60].

Our results support a model that dictates when single carbon equivalents are limited, metabolism of Se is shunted toward pathways requiring the least input of methyl groups, e.g. formation of Se-SUG at the expense of TMSe⁻. Anticarcinogenic [61] selenoamino acids may be precursors to the excretory products Se-SUG and TMSe⁻, as the former increased in liver upon inhibition of methylation capacity. Se-metabolites can modify protein thiols [62,63], and the current study extends those observations by suggesting increased protein thiol modification accompanies reduced methylation capacity. We show an accumulation of tissue Se that could not be accounted for as low molecular weight Se or specific selenoprotein expression, and observed a greater effect of POA treatment on concentrations of total Se in liver than in kidney. This differential effect on liver vs. kidney is concordant with the findings of Tandon et al. [44] and suggestive that liver may be more metabolically active toward Se than kidney.

Both age and obesity may induce declines in capacity for methylation of Se, as the inhibitor of methylation SAH increases with age [64–66]. Older rats have a decreased capacity for TMSe⁻ formation relative to young ones, but are unimpaired in their ability to produce Se-SUG [67]. Additionally, obese subjects exhibit impaired hepatic transmethylation [68] and exhibit increased levels of SAH [69]; although effects of obesity on metabolism of low molecular weight Se are unknown, body mass index is correlated with levels of the predominate plasma selenoprotein, SEPP1 [34]. In this context, our study suggests that alterations in hepatic or renal SAM/SAH can alter the metabolism of Se in individuals consuming apparently adequate amounts of that nutrient.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2013.04.007.

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