

Rumen Microorganisms Decrease Bioavailability of Inorganic Selenium Supplements

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Abstract Despite the availability of selenium (Se)-enriched trace mineral supplements, we have observed low Se status in cattle and sheep offered traditional inorganic Se supplements. Reasons for this may include inadequate intake or low bioavailability of inorganic Se sources. The objective of this study was to determine whether rumen microorganisms (RMO) alter the bioavailability of Se sources commonly used in Se supplements. Rumen microorganisms were isolated from ewes ($n = 4$) and incubated *ex vivo* with no Se (control), with inorganic Na selenite or Na selenate, or with organic selenomethionine (SeMet). Total Se incorporated into RMO and the amount of elemental Se formed were determined under equivalent conditions. Incorporation of Se from Na selenite, Na selenate, or SeMet into RMO was measured as fold

change compared with control (no added Se). Incorporation of Se into microbial mass was greater for SeMet (13.2-fold greater than no-Se control) compared with inorganic Se supplements ($P = 0.02$); no differences were observed between inorganic Na selenate (3.3-fold greater than no-Se control) and Na selenite (3.5-fold greater than no-Se control; $P = 0.97$). Formation of non-bioavailable, elemental Se was less for RMO incubated with SeMet compared with inorganic Se sources ($P = 0.01$); no differences were observed between Na selenate and Na selenite ($P = 0.09$). The clinical importance of these results is that the oral bioavailability of organic SeMet should be greater compared with inorganic Se sources because of greater RMO incorporation of Se and decreased formation of elemental Se by RMO.

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Introduction

Selenium (Se) has been recognized for years as an essential trace element for ruminant animals. In general, the majority of livestock raised in low-Se regions do not receive sufficient dietary Se for optimum health. Severe Se deficiency in ruminants results in nutritional myodegeneration known as “white muscle disease” [1], whereas insufficient Se intake has been implicated as the cause of a group of Se-responsive disorders including unthriftiness, reduced weight gain, and immunosuppression [2]. The Se status of plants, animals, and humans varies markedly around the world as a result of different geological conditions. Animal health is affected by Se deficiency in the diet, which depends on the amount of bioavailable Se taken up by plants [3]. In the USA, a survey of state veterinarians and state veterinary diagnostic labs revealed that Se

deficiency diseases were diagnosed in 46 states and were reported to be an important livestock problem in regions of 37 states [4]. Selenium is incorporated into selenoproteins whose functions range from antioxidant, anti-inflammatory, and detoxification to thyroid hormone activation [5].

Livestock fed Se-deficient forages must receive Se supplements to ensure optimum health. In the USA, Se was approved as a feed additive in 1979 by the Food and Drug Administration (FDA) to respond to the documented deficiency of Se in animal feeds, first at a concentration of 0.1 mg/kg of dry matter (DM), and then after April 1987 at a concentration of 0.3 mg/kg of DM [6]. Selenium supplementation is credited with improving animal health, e.g., reduced prevalence of retained fetal membranes, decreased severity and prevalence of clinical mastitis, decreased somatic cell counts in milk, and decreased calf mortality (reviewed in [7]), and increased mobilization and killing capacity of neutrophils [8–11].

There has been a significant decline in nutritional myodegeneration cases in ruminant species using the current FDA-allowed Se supplementation regulations. Nonetheless, we have observed suboptimal blood-Se concentrations in ruminants offered Se supplements [12]. Reasons for this may include inadequate Se intake or problems with Se bioavailability. Selenium bioavailability can be limited by dietary factors. For example, increasing dietary sulfur decreases Se bioavailability, and the presence of cyanogenic glycosides in certain legumes are also antagonistic to Se (reviewed in [13]).

There is also large variation in oral bioavailability between different chemical forms of Se. Most Se is supplemented in the form of inorganic Na salts in mineral mixtures that are commonly left outside for several weeks for sheep or cattle to consume ad libitum. The primary form of Se in these supplements is inorganic Na selenite because it is easier to purchase than Na selenate, although Na selenate is more stable than Na selenite and has greater small intestinal absorption in nonruminants (reviewed in [14]). Selenium can also be supplemented as organic selenomethionine (SeMet). In the USA, organic Se was approved as a feed additive in 2003 by the FDA at the same supplementation rates as inorganic Se forms, even though there is a documented increase in bioavailability.

Rumen microorganisms (RMO) may decrease Se bioavailability by reducing selenite into non-absorbable elemental Se, which is then excreted in the feces [15–17]. It is known that absorption of Se by ruminants is less compared with nonruminants [18]. For example, researchers have shown that absorption of orally administered Se was 34 % in sheep compared with 85 % in swine [13]. Although enrichment of Se occurs in RMO compared with dietary levels ([19, 20], smaller amounts of inorganic Na selenite or Na selenate are incorporated into RMO than SeMet, the primary form of Se found in Se yeast [21, 22] and high-Se grains and forages [14, 23].

We previously showed that ewes receiving Na selenite or Na selenate by weekly oral drenching had decreased whole-

blood and serum Se concentrations compared with ewes receiving the same dosage of SeMet as Se yeast [24]. To determine if RMO were responsible, in part, for these findings, we conducted an ex vivo experiment to evaluate the effect of Na selenite, Na selenate, and SeMet on Se uptake and elemental Se formation by RMO. We hypothesized that RMO would incorporate more Se from SeMet compared with Na selenite or Na selenate and would form less elemental Se from SeMet compared with either inorganic Se salt.

Materials and Methods

Experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Oregon State University. The experimental design is a split-plot design, in which RMO from individual ewes are the whole-plots and Se sources are the subplots. Adult Suffolk crossbred ewes ($n = 4$) were used to collect rumen fluid during the summer months. Ewes were receiving dietary Se from forage, grass or alfalfa hay, and from trace mineral supplements that contained Se. Ewes were sedated and an orogastric tube placed to obtain 400 mL of rumen fluid. The fluid was placed in an air tight container until further processed. Each sample was processed on the day of collection.

Concentrations of Se in the forage from the pastures these ewes were grazing ranged from 0.12 to 0.14 $\mu\text{g/g}$ of DM. Assuming pasture DM intake of 2 % of body weight, ewes would consume between 0.12 and 0.26 mg of Se/day [24]. The Se concentration of the grass hay and alfalfa hay were 0.02 and 0.05 $\mu\text{g/g}$ of DM, respectively [24]. These feed sources contributed less than 20 μg of Se/day. The mineral supplement these ewes consumed contained 200 mg/kg of Se. For an average mineral intake of 8 g/day, an additional 1.6 mg of Se would be consumed. Thus, Se intake ranged from 1.72–1.86 mg/day in these four sheep.

The procedure for isolating RMO from rumen fluids described by Whanger et al. [20] was followed with minor modifications. Rumen contents were strained through 3 to 4 layers of cheese cloth into a 1000-mL beaker, which was then sealed. Rumen contents were stirred to evenly distribute rumen fluid. Next, 40-mL aliquots of rumen fluid were placed into eight 50-mL polypropylene centrifuge tubes (ISC Bioexpress, Kaysville, UT). These tubes were then centrifuged for 10 min at a relative centrifugal force (RCF) of $750\times g$ in a Beckman TJ-6 centrifuge with swinging bucket rotor to sediment debris. The supernatants containing RMO were removed and pooled. Thirty-milliliter aliquots of this fluid were placed into four 50-mL polypropylene centrifuge tubes (Thermo Fisher Scientific Inc., Waltham, MA) for additional washing of RMO. All tubes were centrifuged at a RCF of $10,000\times g$ in a Sorvall RC5B centrifuge (Thermo Fisher Scientific Inc.) for 20 min to sediment RMO. Supernatant was discarded and

5 mL of nitrogen-gassed phosphate-buffered saline (PBS), and three zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK) were added to each tube. Contents were re-suspended using a vortex. Once re-suspended, another 20 mL of nitrogen-gassed PBS was added to each tube and vortexed. Tubes were centrifuged again at a RCF of $10,000\times g$ in the Sorvall RC5B centrifuge for 20 min. The supernatant from all centrifuge tubes was discarded, and the centrifuge tubes were weighed to calculate RMO mass (g) by subtraction of original dry weight of centrifuge tubes. Then, 5 mL of nitrogen-gassed PBS was placed into each tube, and pelleted RMO were re-suspended as previously described.

Next, RMO were incubated with different Se sources. All four treatments were applied to each ewe's RMO. One milliliter of solution containing 1.5 mg/kg of Na selenite (767.1 mg $\text{Na}_2\text{SeO}_4/\text{dL H}_2\text{O}$; RETORTE Ulrich Scharrer GmbH, Röthenbach, Germany) was added to one tube, 1 mL of solution containing 1.5 mg/kg Na selenate (1.571 g $\text{Na}_2\text{SeO}_3/\text{dL H}_2\text{O}$; RETORTE Ulrich Scharrer GmbH) was added to one tube, 1 mL of solution containing 1.5 mg/kg SeMet (868.0 mg $\text{C}_5\text{H}_{11}\text{NO}_2\text{Se}/\text{dL H}_2\text{O}$; Acros Organics, Thermo Fisher Scientific) was added to one tube, and 1 mL of distilled water (control) was added to one tube of microbial suspension. Preliminary experiments performed in our laboratory had shown that the optimum amount of Se needed for maximal bacterial growth (most gas production and highest optical density readings, thus elemental Se formation) for each Se source occurred between 1 to 1.5 mg/kg Se over the range of 1 to 3 mg/kg Se added (data not shown). These Se concentrations were similar to those previously reported for incubations of Se with RMO [21]. Next, 25 mL of nitrogen-gassed PBS was added to each tube and the contents were re-suspended by vortexing. The tubes were aerated with USPS grade nitrogen (Airgas, Corvallis, OR) for 5 min, and a rubber stopper was placed over each tube. All tubes were placed at rumen temperature (42°C) in an incubator for 24 h.

Total Se incorporated into microbial mass was then determined. After incubation, each tube was centrifuged for 20 min at a RCF of $10,000\times g$ in the Sorvall RC5B centrifuge. Supernatant was removed, and the microbial pellets were re-suspended in 1.5-mL PBS and mixed using a vortex. Then, 20 mL of a 50 % ethanol/PBS solution was added to dissolve any remaining nonbound Se (selenite, selenate, or SeMet) and tubes were again vortexed. Tubes were centrifuged for 20 min at a RCF of $10,000\times g$ in the Sorvall RC5B centrifuge, and the supernatant containing residual nonbound Se (selenite, selenate, or SeMet) was discarded. Pelleted RMO and elemental Se were re-suspended in 1.5 mL of PBS, followed by vortexing. Each of the four samples was then split into two paired samples. One sample from each of the four pairs was placed into a 1-mL storage tube and frozen at -80°C . The other sample from each of the four pairs was placed into a 50-mL polypropylene centrifuge tube for further processing.

To these tubes, 20 mL of PBS containing 750 mg of ammonium polysulfide (gift from Tessenderlo Kerley Inc., Phoenix, AZ) was added to dissolve elemental Se (reviewed in [22, 25, 26]). The tubes were then centrifuged for 20 min at a RCF of $10,000\times g$, and the supernatant containing dissolved elemental Se was discarded. Next, 0.75 mL of PBS was added to each sample and vortexed. The samples were placed in 1-mL centrifuge tubes and frozen at -80°C . Paired samples were submitted for total Se analysis to the Center for Nutrition, Diagnostic Center for Population and Animal Health, Michigan State University (East Lansing, MI), by ICP-MS methodology. The difference between paired samples was equal to the amount of elemental Se formed. Total Se present in a sample after ethanol and ammonium polysulfide extraction represented Se incorporation into microbial mass, which was expressed as ng Se/mL/g RMO.

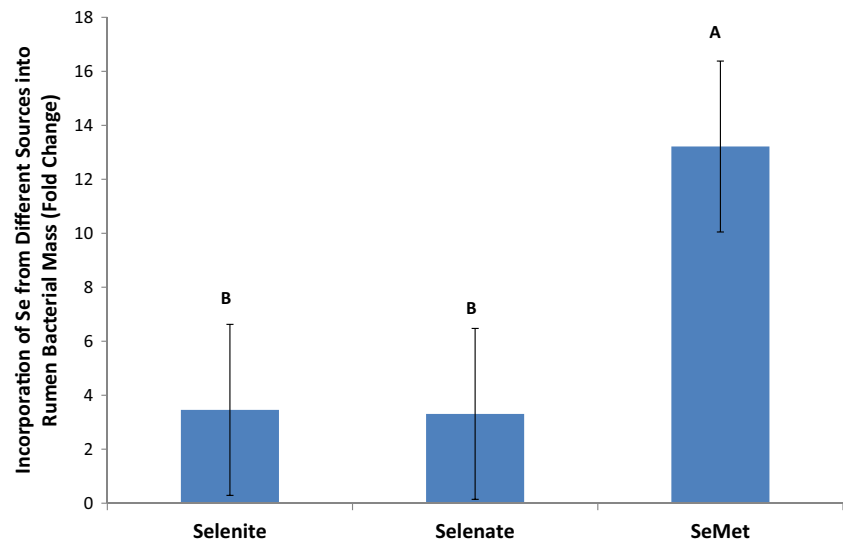
Statistical analyses were performed in PROC MIXED using SAS, version 9.1 (SAS, Inc., Cary, NC, USA) software. Prior to analysis, all data were converted to fold change compared with no Se control to account for the variation between ewes. The exception was proportion of total Se incorporated into RMO. To achieve normality, the data for elemental Se formation were natural log-transformed. Using mixed-model analysis, the statistical model included chemical form of Se (Na selenate, Na selenite, and SeMet) as fixed effect and ewe as random effect. A priori, orthogonal contrasts were calculated to determine differences between organic and inorganic Se sources (SeMet vs. Na selenate and Na selenite combined) and among inorganic Se sources (Na selenate vs. Na selenite). Data are reported as means \pm SEM for Se incorporation into bacterial mass and proportion of total Se incorporated into RMO and as geometric means \pm SEM for elemental Se formation. Significance was accepted at $P \leq 0.05$.

Results

Incorporation of Se from Na selenite, Na selenate, or SeMet into RMO mass was measured as fold change compared with control (no added Se). When RMO were incubated with inorganic Se sources, the incorporation of Se into RMO was essentially equivalent for Na selenite (3.5 ± 3.2 -fold greater than no-Se control) or Na selenate (3.3 ± 3.2 -fold greater than no-Se control) ($P = 0.97$; Fig. 1). In contrast, the incorporation of Se into RMO was greater for SeMet (13.2 ± 3.2 -fold greater than no-Se control or $3.8/4.0$ times greater than Na selenite/Na selenate) compared with inorganic Se sources ($P = 0.02$).

Formation of elemental Se by RMO under ex vivo incubation conditions was measured as fold change of various Se sources compared with no-Se-added control. The formation of elemental Se by RMO was similar for Na selenite (73 ± 16 -fold greater than no-Se control) or Na selenate (39 ± 9 -fold greater than no-Se control) ($P = 0.09$). In contrast, the formation of elemental Se

Fig. 1 Incorporation of selenium (Se) from Na selenite, Na selenate, or selenomethionine (SeMet) into rumen bacterial mass compared with control (no added Se). Fold change is expressed as ng Se/mL/g bacterial mass. Data are shown as mean \pm SEM, with *letters* indicating significant differences. The incorporation of Se into rumen bacterial mass was significantly greater ($P = 0.04$) for SeMet compared with either inorganic Se source



by RMO was less for SeMet (21 ± 5 -fold greater than no-Se control) compared with inorganic Se sources ($P = 0.01$).

The proportion of total Se incorporated into RMO was similar for Na selenite (3.3 ± 1.6 %) and Na selenate (5.5 ± 1.6 %; $P = 0.15$). In contrast, the proportion of total Se incorporated into RMO was greater for SeMet (23.3 ± 1.6 %) compared with inorganic selenocompounds ($P < 0.0001$).

Discussion

We have shown that isolated RMO supplemented with inorganic Na selenite or Na selenate *ex vivo* incorporate Se into bacterial proteins to a similar extent. This is consistent with our previous *in vivo* findings in which ewes receiving Na selenite or Na selenate once weekly as an oral drench at the maximum FDA allowed concentration had similar whole-blood and serum Se concentrations [24]. In contrast, when RMO were supplemented with organic SeMet *ex vivo*, the incorporation of Se into rumen bacteria was 3.8 to 4 times greater for SeMet than for inorganic Se sources. This is consistent with our previous *in vivo* studies whereby we showed that the maximum FDA allowed concentration of SeMet supplementation was equally effective as supranutritional rates (i.e., 3 to 5 times greater) of inorganic Se supplementation in increasing whole-blood Se concentrations, demonstrating the greater bioavailability of oral organic Se [24]. Others agree that bioavailability of Se from inorganic selenite and selenate is similar in ruminants [27, 28]. Serra et al. [29] showed equal apparent absorption and retention of selenite and selenate in wethers when supplemented at 0.2 mg Se/kg dietary dry matter.

A large percentage of amino acids liberated by microbial proteolysis in the rumen are reutilized for microbial protein synthesis. SeMet is not absorbed *in situ* to any appreciable extent in the rumen [30] and neither are the inorganic Se

sources [18]. Rumen microbes are capable of metabolizing inorganic ^{75}Se and incorporating it into microbial protein [31]. Bacteria reduce selenate (SeO_4^{-2}) through selenite (SeO_3^{-2}) to elemental Se (Se^0) [32]. Selenium can also become incorporated into proteins as part of the amino acids selenocysteine (SeCys) or SeMet. This occurs as a result of the reduction of selenite with reduced glutathione to make selenodiglutathione, which is further reduced to glutathioselenol (GS-SeH), which is further reduced to hydrogen selenide (H_2Se) providing the necessary reactive intermediates for Se incorporation into amino acids, or further reduction to elemental Se [32]. When beef cattle are fed a high Se diet, there is an increase in the number of Se-reducing microbes [33]. In this study, sheep consumed a Se-rich mineral supplement, which when combined with feed sources provided 1.72 to 1.86 mg Se/day. This may have increased elemental Se formation by RMO. Future research is warranted to determine if dietary Se status of ruminants (Se rich, replete, or deplete) alters Se incorporation into RMO and elemental Se formation.

Post ruminal physiologic absorption mechanisms of ruminants and monogastric animals in the small intestine are similar [18]. Lysis of RMO begins in the abomasum by way of lysozyme in abomasal secretions. Microbes yield protein of high biological value, as protein content is approximately 27 and 45 % of total DM for bacteria and protozoa, respectively [34]. Thus, Se incorporated as SeMet into bacterial proteins is readily available for absorption in the small intestine.

Our results are consistent with others [22] who showed that uptake of ^{75}Se from [^{75}Se]selenite by RMO in sheep ranged from 21 to 34 %, whereas uptake of ^{75}Se Met by RMO was greater, ranging from 35 to 43 %. Chromatography of the hydrolysate of RMO revealed that ^{75}Se was present predominantly as SeCys (42 %) vs. SeMet (20 %) when incubated with [^{75}Se]selenite but present predominantly as SeMet (79 %) when incubated with ^{75}Se Met. However, the hydrolysis

recovery was much less in RMO incubated with selenate (51 %) than those incubated with SeMet (84 %). Similar results were observed by Mainville et al. [35] when comparing Se uptake of inorganic ^{82}Se and organic ^{77}Se Sel-Plex[®] by bovine RMO. Organic Se was five times better retained by RMO in cattle than the inorganic Se source [35]. Thus, RMO are capable of taking up selenite and incorporating it into seleno-amino acids, but do so to a lesser extent than they uptake SeMet, which remains as SeMet in bacterial proteins.

Rumen microbes also contribute to the conversion of dietary Se to unavailable forms and its eventual loss in the feces as insoluble elemental Se [27]. For example, Serra et al. [29] showed that most of the Se in ruminal fluid of supplemented wethers was insoluble, demonstrating the negative influence of the rumen environment on Se bioavailability. We showed in our ex vivo experiments that more elemental Se was formed from inorganic Se sources compared with SeMet. This is consistent with the report that absorption of Se in ruminants is less efficient than that in monogastric animals [13], apparently because of the conversion of inorganic Se by RMO to unavailable forms [20]. A possible reason for this inefficiency of absorption may relate to the rumen environment. The normal rumen pH is roughly 6.2, thus mildly acidic. Based on the type of feed a ruminant ingests, the pH of the rumen may become more acidic, especially when fed high-starch diets. Under acidic conditions, oxidized and biologically active selenite may be reduced to elemental Se and, therefore, no longer biologically available to the animal [8]. Inorganic forms of Se undergo reductive metabolism through a number of intermediate steps leading to the generation of hydrogen selenide (H_2Se), which serves as the precursor for the synthesis of essential selenoproteins [14]. In mammalian cells, reductive metabolism occurs intracellularly for selenate, and glutathione protects selenate from further reduction to elemental Se. This may not be true for bacterial cells, based on our results showing similar production of elemental Se for both inorganic Se sources. SeMet is also metabolized intracellularly to the same key intermediate H_2Se ; however, SeMet can also be incorporated into bacterial proteins directly and thus is protected from further metabolism. Conversely, reduction of inorganic Se sources leads to production of elemental Se, which is nonabsorbable in mammals.

Selenium, if present in plant-based feeds, is normally found in organic forms, e.g., as SeMet, SeCys, or SeMethylselenocysteine [14]. The inorganic forms of Se, selenite and selenate, are found much less frequently in plants and normally in very low amounts [14], unless they are Se accumulator plants in which case selenate concentrations will be high. Of the organic forms, SeMet is the major selenocompound in cereal grains and enriched yeast; SeMethylselenocysteine is a major selenocompound in Se accumulator plants [36] and some plants of economic importance such as garlic and broccoli exposed to excess Se. Both organic and inorganic forms of Se can

be utilized in the body to produce selenoproteins, but in the ruminant, RMO influence their bioavailability for later absorption in the intestinal tract.

Thus, the use of organic Se for Se supplementation in ruminants has shown greater tissue Se uptake and greater milk and blood concentrations of Se when compared with inorganic Se sources [37]. When comparing the bioavailability of inorganic Se sources, selenite and selenate appear to produce similar whole-blood Se concentrations [24, 28] because microbial metabolism of the inorganic Se sources is similar.

Our original hypothesis that selenate would be more bioavailable than selenite at supplemental doses does not appear to be true in ruminants, based on either ex vivo experiments reported here, or whole-animal experiments reported previously by us [24] and others [27–29]. The majority of both inorganic Se sources are converted into nonabsorbable forms in the rumen and, thus, inorganic selenate and selenite are similarly unavailable for absorption in the small intestine.

In conclusion, we showed that organic Se as SeMet was incorporated to a greater extent into RMO than inorganic Se sources and resulted in less elemental Se formation. Decreased oral bioavailability of inorganic Se compared with Se yeast noted in our whole animal studies may be explained by these ex vivo results showing increased elemental Se formation and decreased microbial incorporation of inorganic Se sources. In ruminants, the improved bioavailability of SeMet compared with inorganic Se may be the result of rumen-based reactions (i.e., increased incorporation of SeMet into RMO and decreased formation of elemental Se) rather than at the level of the small intestine (i.e., increased absorption efficiency). Consumption of Se-fertilized forage [38, 39] as a source of organic Se provides an attractive alternative to inorganic Se supplements, because organic SeMet in forage would be better incorporated into RMO and would result in less elemental Se formation.

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