Toxicologic Evaluation of Chronic Selenosis in Wyoming Herbivores

Abandoned Coal Mine Lands Research Program

Final Report
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Historical Rationale

Much of the western Great Plains, including large areas of Wyoming, is underlain by selenium (Se) bearing shales. Where these shales are exposed to weathering, Se may undergo chemical changes which result in bioaccumulation by forage plants and ultimately poisoning in grazing herbivores. The potential for bioaccumulation is further exacerbated by the region's meager rainfall and alkaline soils. A similar biogeochemical process is theoretically possible when seleniferous materials associated with coal seams are disturbed and then replaced during surface mine reclamation (Boon, 1989). Not surprisingly, the soil Se threshold which initiates this series of events is of concern to regulatory agencies and the mineral industry.

Ultimately, permissible soil and spoil Se concentrations in reclaimed coal mine lands hinge on the potential for toxic effects in grazing animals. When our group initially began investigating naturally-occurring chronic Se poisoning (selenosis) in 1989, the prevailing dogma was based upon early (1930-1960) reports from the experiment stations at Wyoming and South Dakota (Beath et al., 1934; Franke, 1934a; Franke, 1934b; Franke & Potter, 1934; Draize and Beath, 1935; Trelease and Beath, 1949; Rosenfeld and Beath, 1964). However, personal experience and anecdotal information from ranchers and veterinarians in northeastern Wyoming and the Nebraska panhandle suggested that large pieces of this dogma were *qualitatively* inaccurate (Raisbeck et al., 1993; Edmondson et al., 1993; O'Toole et al., 1996) and, as a result, possibly even further off the mark quantitatively. Thus, although the experiments of this study were not undertaken with the specific goal of establishing a magic "level" *per se*, they should provide a sound experimental basis for extracting such information from soil-plant accumulation experiments, epidemiologic data in animals and the older scientific literature.

Project Overview

Model: In order to evaluate the toxicity of any substance, one must first define the target species. In keeping with the objective of returning reclaimed mine lands to livestock and wildlife grazing we selected cattle and antelope as experimental models. These species are: 1) among the most numerous and economically important herbivores in coal mining areas of Wyoming; 2) lend themselves to experimentation; and 3) make good experimental surrogates for other large prairie herbivores such as deer and elk. Although subsequent work outside of the AML-funded project suggests that horses may be more sensitive to chronic selenosis than ruminants, they were originally excluded because of expense and the fact that, under normal management, they aren't as completely dependent on forage for nutrition. Swine, although exceptionally susceptible to selenosis, were excluded because they are unlikely inhabitants of the post-mining environment in Wyoming.

Toxicant(s): Selenium occurs in a variety of chemical forms in the environment. Prior to 1991 most experimental studies of selenosis in large herbivores utilized inorganic Se salts such as selenite ($SeO_3^{=}$) (eg. Miller and Williams, 1940; Maag et al., 1960; Glenn et al., 1964; Olson and Embry, 1973; Ahmed et al., 1990); however the predominant form of Se in forage plant species is thought to be Se analogs of S amino acids, especially selenomethionine (Semet) (Olson, 1978). As noted by Olson (1978) "evaluation of the toxicity of the plant

based on its total selenium is hardly possible". There is a body of data in laboratory rodents (Waschulewski and Sunde, 1988) and waterfowl (Heinz et al., 1988; Fairbrother and Fowles, 1990) which suggests that Semet accumulates to a greater extent than inorganic Se and may be more toxic than inorganic Se salts. Thus the thrust of our initial experiments was to: 1) determine if and to what degree Semet was synthesized from inorganic forms by the rumen microflora and 2) compare the bovine toxicity of Semet to inorganic Se salts which have been historically used in herbivore experiments. The results of these experiments were to be validated in a second species of primary consumer (antelope), with similar diets. Logistic problems forced us to substitute a seleniferous hay diet at the latter point (see below). Finally, we attempted to use the characteristic endpoints determined from these studies to predict the potential hazard of "naturally" seleniferous forages from native pastures and reclaimed mine lands.

In Vitro Experiments

It is impossible to divorce the effects of any toxicant on a grazing ruminant from its interaction with the rumen microflora. There are numerous examples of agents whose toxicity is potentiated (eg. nitrate, urea) or blocked (eg. penitrem, ochratoxin) by rumen fermentation. Indeed, it has been suggested that orally administered nutritional Se supplements are reduced to a water-insoluble form, probably selenide, by ruminants (Oldfield et al., 1994). The ruminant digestive process may itself be a target of toxicant action. Toxic agents such as crude oil which inhibit rumen function may result in malnutrition and even death of the host herbivore. Ill-thrift or poor productivity is a common, if ill-defined condition attributed to Se by the historical literature. We hypothesized that the latter condition might be accounted for by direct Se-toxic effects on rumen microbial function. To test this hypothesis, and to characterize the ruminal metabolites of Se, we exposed normal bovine rumen microflora to Se *in vitro*.

Model (rumen metabolism): Rumen inoculum was obtained via fistula from cattle maintained on grass hay diets containing approximately 0.3 ppm Se. Culture conditions were as described previously (Siemion et al, 1992) with the addition of an activated charcoal cartridge on the system to trap evolved gas (Fig. 1). Sufficient [^{75}Se]-Na_2SeO_3 was added to each artificial rumen culture to provide 0, 0.1, 1.0, 10 or 50 ug supplemental Se per g dry matter. After incubating for 24 hr, the culture was killed by freezing, treated with sodium dodecylsulfate and separated into insoluble and soluble fractions by centrifugation. The soluble fraction was further separated into protein and sub-protein fractions by filtration[1]. The insoluble fraction was incubated with acid pepsin to simulate abomasal digestion (Tilley and Terry, 1963; Siemion et al., 1992) and again separated into soluble and insoluble fractions by centrifugation. The Se content of each fraction was determined by counting ^{75}Se. The various fractions then were derivatized with iodoacetate, hydrolyzed with HCl where appropriate and analyzed by thin layer chromatography/radioautography (Fig 1).

Results (rumen metabolism): Very little if any ^{75}Se was trapped on the activated charcoal trap indicating that few if any volatile Se compounds were formed by rumen fermentation.

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[1] Amicon PM-30, Amicon Corp, Lexington, MA

Only about 10% of the total added ^{75}Se was incorporated into soluble proteins (as determined by filtration) in the artificial rumen and 10%-20% appeared in the low molecular weight, soluble fraction. The majority (60%-70%) of added ^{75}Se remained with the water insoluble sediment after 24 hr incubation with rumen microflora and treatment with SDS. Approximately 25% of added ^{75}Se remained with the insoluble fraction even after acid pepsin treatment of the initial fermentation residue. The soluble fractions, after hydrolysis and derivitization yielded 6 distinct compounds by TLC, some of which matched authentic standards of selenocystine, selenomethionine and $SeO_4^=$.

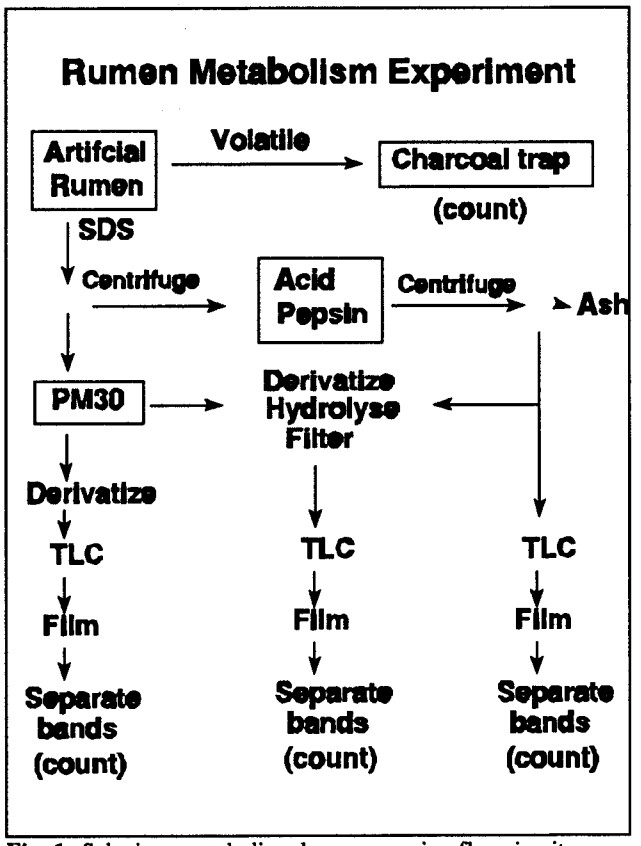

Fig. 1 Selenium metabolism by rumen microflora *in vitro*.

Despite extensive precautions we took to avoid such a possibility, the $SeO_4^=$ ion is probably an artifact of the extraction and derivatization process. It does not seem reasonable that [^{75}Se]-$SeO_3^=$ would be further oxidized in the fundamentally reducing rumen environment. Whether this ion was derived from $SeO_3^=$ which escaped microfloral metabolism or by oxidation of other Se compounds during the analytical process is unknown. The water soluble, low molecular weight fraction of the initial fermentation appeared to consist mainly of small polypeptides, at least some of which contained ^{75}Se as selenocysteine or selenomethionine. We identified what we believe to be selenodiglutathione, but were unable to obtain an authentic standard to confirm this result.

Our original plan was to repeat the experiments with [^{75}Se]-Semet and compare the results with $SeO_3^=$. Unfortunately, the coinvestigator responsible for synthesis of Semet dropped out of the project and we were unable to compare the metabolism of Semet with $SeO_3^=$. We couldn't discern any marked differences in how different concentrations of added ^{75}Se were handled by the rumen microflora. This may have been due (in part) to the imprecision of our experimental method, and to the fact that inoculum was obtained from cattle maintained on normal Se diets. *In vitro* methods such as we used are, of necessity, too short-lived to permit much microbial adaptation. When we repeated the experiment with inoculum obtained from a steer maintained on 25 ppm Se hay a greater percentage of Se from the highest (10, 50 ug/gm) treatments appeared in both the insoluble residue of the fermentation and acid pepsin digest, and as Semet in the soluble fractions. The former would be less bioavailable to the host, the latter potentially more so. Thus, while it appears that rumen microbes are capable of adapting to increased dietary Se, the effect of such adaptation is unclear from these experiments.

Method (effects on digestion): The effects of $SeO_3^=$ or Semet on *in vitro* digestibility, nitrogen (N) utilization and volatile fatty acid (VFA) production were evaluated in a similar model. Selenomethionine or $SeO_3^=$ (0, 0.1, 1.0, 10.0 or 50.0 ug/g dry matter (dm)) were added to a simple artificial rumen similar to that described above which was then incubated at 38C for either 24 or 48 hr. Digestibility (a broad measure of nutrient bioavailability) and residual N (an indirect measure of protein metabolism) were evaluated by measuring the disappearance of biomass and N from the system (Tilley and Terry, 1963). Volatile fatty acid production was measured by extracting the fermented liquor with diethyl ether and quantifying acetate, propionate and butyrate against authentic standards with GC/MS. Each experiment was replicated at least 6 times.

Results (effects on digestion): There were no measurable differences between the effects of similar doses of added $SeO_3^=$ and Semet in the parameters measured. At 24 hr the highest doses (10, 50 ug) of added Se decreased digestibility, but by 48 hr digestion in these cultures surpassed controls (Fig. 2). The latter probably represents a compensatory response as microflora adapted to the presence of Se. Volatile fatty acid (acetate, propionate and butyrate) production was unaffected at 24 hr, but markedly depressed by all doses of Se at 48 hr (Fig. 3). Conversely, N utilization was increased by Se at both 24 and 48 hr by all but the highest dose of Semet (Fig. 4). The latter two results represent a Se-induced shift in the fundamental direction of forage metabolism by the rumen microflora. The net health effects of

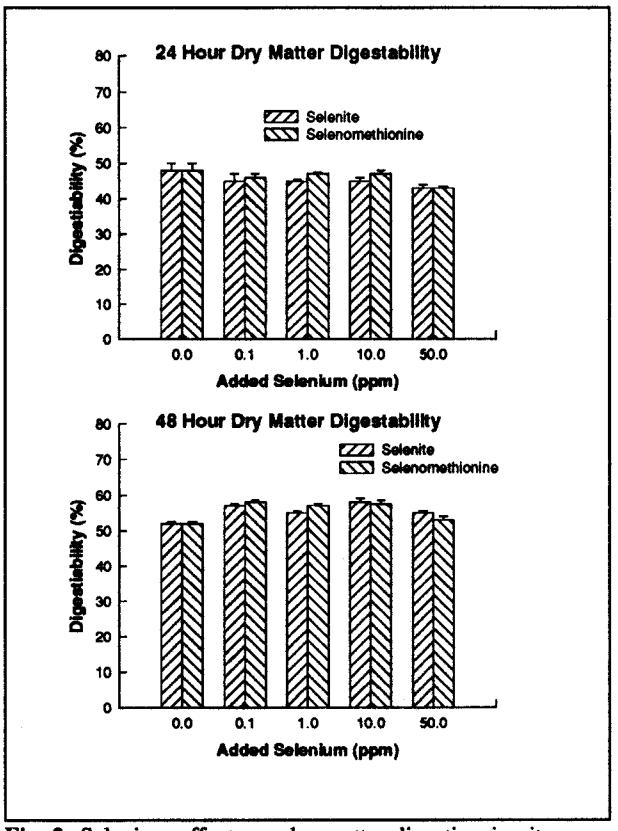

Fig. 2 Selenium effects on dry matter digestion *in vitro*.

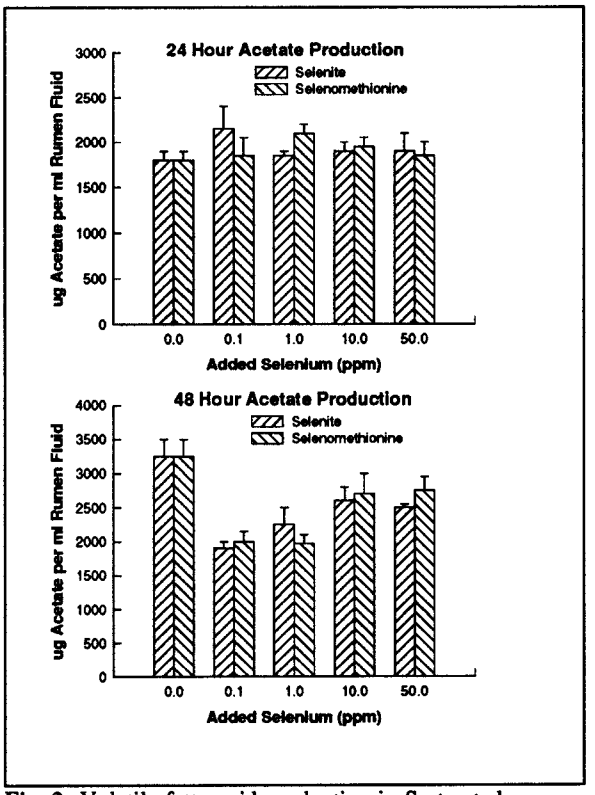

Fig. 3 Volatile fatty acid production in Se treated rumen microflora. Propionate and butyrate were similarly affected.

such a shift would depend on other dietary and environmental factors which aren't possible to simulate with this model.

Lay summary - in vitro experiments:

1) The absence of evolved ^{75}Se rules out dimethylselenide as a rumen metabolic product.
2) A finite portion of dietary $SeO_3^{=}$ is reduced to a form that will likely pass through the GI tract unabsorbed. We weren't able to repeat this experiment with Semet as originally intended, but there a similar effect with nutritional doses of Semet in lambs has been reported (Ehlig et al., 1967). This reduction may account for the apparent resistance of ruminant species to selenosis as compared to monogastrics such as swine or birds.

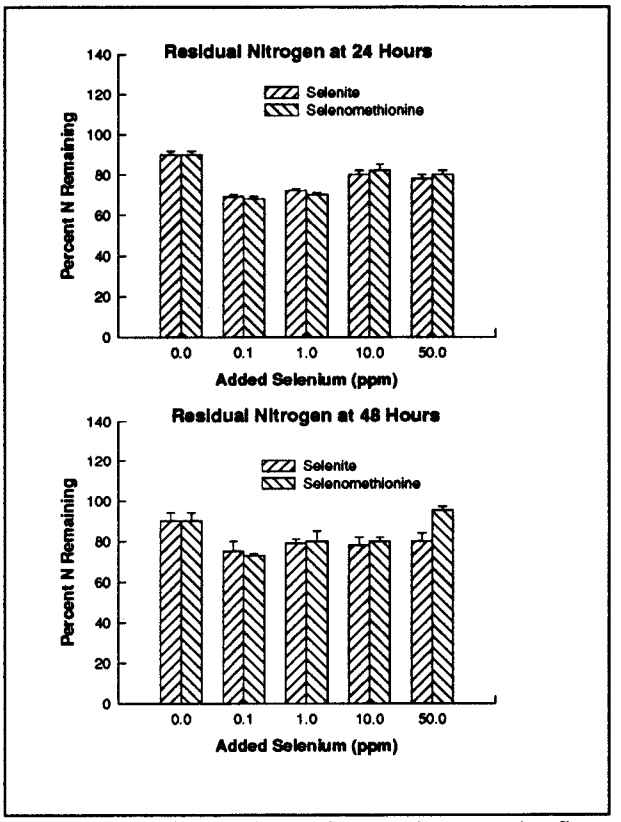

Fig. 4 *In vitro* N utilization by Se-treated rumen microflora.

3) Dietary $SeO_3^{=}$ will, in all likelihood, be presented to the host herbivore as a number of different Se compounds, including Semet. We were unable to directly test the hypothesis that other Se compounds, specifically Semet, are also interconverted between different chemical forms. However this observation seems to preclude practical exposure standards for grazing ruminants based upon speciation of dietary Se.
4) Selenium has both positive and negative effects on rumen microbial fermentation *in vitro*. Neither effect is sufficiently clear cut to draw broad extrapolations to the whole animal. The net result on animal health will depend on ancillary dietary factors such as the ratio of digestible energy to total protein and is thus impossible to generalize from these experiments.

Steer Experiments

Model: Twenty native Wyoming yearling Hereford steers were obtained from the Medicine Bow area and acclimated to a basal diet of finely chopped grass hay nutritionally similar to native grass pastures in the Powder River basin and which contained approximately 0.3 ppm Se. Steers were housed in groups of 4 in concrete floored corrals with a 3 sided shed for shelter. Each steer was vaccinated for common infectious diseases of yearling cattle[2,3],

[2] Bar-Vac®7/Somnus, Anchor Division, Boehringer Ingelheim Animal Health, Inc., St. Joseph, MO.
[3] Horizon®4, Miles Inc., Agricultural Division, Animal Health Products, Shawnee, KS.

given an insecticidal ear tags[4] and treated with a broad spectrum antiparasitic compound[5]. Each steer was fitted with an electronic key[6] on a neck chain which permitted access to a similarly keyed feeder containing his assigned diet. Salt (a 1:1 mixture of NaCl and sodium polyphosphate) and water (<50 ppb Se) were provided *ad lib*. After a 45 day acclimation period, steers were randomly assigned to a control group (basal diet) or to one of 6 experimental groups fed either sodium $SeO_3^{=}$[7] (0.15, 0.28 or 0.8 mg Se/kg body weight (bw)) or Semet[8] (0.15, 0.28 or 0.8 mg Se/kg bw). Selenomethionine purity was confirmed (>99%) by elemental analysis[9], fluorometry (Raisbeck et al., 1996) and thin layer chromatography. Dosages were calculated to approximate 5, 10 or 25 ppm dietary Se exposure under pasture conditions and were adjusted weekly on the basis of the previous week's body weight. Each dose of Se was absorbed into 80 g of ground corn cob and thoroughly mixed by hand with a measured amount (approximately 10 kg) of chopped hay and 250 g ground cottonseed meal daily. Hay consumption was monitored daily and readjusted as needed to insure each steer completely consumed each day's Se dose in a 24 hr period.

Endpoints (general): Each animal was observed several times daily for any evidence of ill-health and weighed weekly. Blood was collected by jugular venepuncture at 21 day intervals for blood and serum Se (Raisbeck et al., 1996), blood glutathione peroxidase (GSHPx) (Agergaard and Jensen 1982) and a complete blood count (CBC, including red blood cell count, white blood cell count, hemoglobin, hematocrit, and differential white cell count). Serum was harvested from blood and analyzed for blood urea nitrogen (BUN), serum albumin, globulin, protein, alkaline phosphatase (AP), glucose, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK) and total bilirubin with an autoanalyzer[10] and alpha-hydroxybutyrate dehydrogenase activity (HBDH) with a commercial kit[11]. Each test was selected to reflect the integrity of one or more organ systems; taken in aggregate this battery was designed to serve as the most extensive real-time monitor of health practical.

Endpoints (immunology): After calves were established on experimental diets for 45 days each was immunized with a single subcutaneous injection (2 ml) of hen egg albumin (OVA, Sigma A5503) 20 mg/ml in potassium alum adjuvant (Proom, 1943). An indirect enzyme linked immunosorbant assay (ELISA) developed in our laboratories (Schamber, 1994) was used to measure OVA-specific antibody (primary antibody response, PAR) in serum collected at the subsequent three bleedings. The PAR is a measure of the ability of the immune system to respond to an unfamiliar challenge, for example a new virus or vaccine. Total immunoglobulin was measured by an antigen-trap ELISA. Lymphocyte blastogenesis was evaluated *in vitro* by stimulating lymphocytes harvested at the same intervals with phytohemagglutinin (PHA), lipopolysaccharide (LPS), concanavalin-A (Con-A). Interleukin 3 (IL3) and 4 (IL4) production were measured. These are assays which measure the ability of

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4 Guard Star Tags, V-Tex, Cody, WY.
5 Ivomec® 1% injection for cattle, Merck Agvet Division, Merck & Co., Inc., Rahway, NJ.
6 Calan Broadbent Feeding System, American Calan Inc., Northwood, NH.
7 Sigma Chemical Co., St. Louis, MO.
8 Calbiochem Bulk Chemicals, La Jolla, CA.
9 Desert Analytics Lab, Tucson, AZ.
10 IDEXX 8008, IDEXX Corp., Westbrook, Maine, USA.
11 Sigma Chemical Co. DG120-K, Sigma Chemical Co., St. Louis, Missouri, USA.

immune blood cells to react to various types of challenge *in vitro*. A detailed description of the methodologies used has been published previously (Schamber, 1994).

Endpoints (pathology): At the end of the 120 day feeding period, each steer was humanely killed and subjected to a complete necropsy followed by a detailed histopathologic review. The weight of each carcass and, following trimming of excess fat and connective tissue, the weights of the following organs were recorded: liver, kidneys, testes, spleen, pancreas, brain, adrenals, thyroids, pituitary, heart, left and right ventricular free walls, and septal myocardium. The following tissues from each animal were stored in 10% neutral buffered formalin for light microscopic evaluation: salivary gland, tongue, esophagus, reticulum, rumen, omasum, abomasum, small bowel (three levels), cecum, colon, liver, gall bladder, pancreas, lung (anterior, middle and posterior lobes of the left lung), heart (right and left ventricles at the papillary muscles, and interventricular septum), thoracic aorta, kidneys, bladder, adrenals, thyroids, pituitary, thymus, spleen, mesenteric and hepatic lymph nodes, testicle, bone marrow (femur), skin (three sites: tail, right lateral thorax, and dorsal midline cervical area), brain, spinal cord, seven skeletal muscles (biceps brachii, extensor carpi radialis, vastus lateralis, biceps femoris, tibialis cranialis, longissimus lumborum and psoas major,) and four peripheral nerves (ulnar, median, ischiatic and fibular nerves). Hooves were sectioned sagittally with a band saw prior to fixation.

Eyes were trimmed of extraocular tissue, fixed in Zenker's-acetic acid (Luna, 1985) and cut in a vertical midsagittal plane through the optic papilla. Following fixation in formalin, the following 11 levels of each brain and seven transverse and longitudinal levels of spinal cord, including spinal nerve roots, were prepared for histology with hematoxylin-eosin and Von Kossa stains (Luna, 1985): mid-sagittal level of cerebellum and septotemporal level of hippocampus; coronal levels of frontal, parietal and occipital cortex, basal ganglia, thalamus; midbrain; medulla oblongata at middle-posterior cerebellar peduncles, obex and near the junction with the first cervical spinal cord segment (C1); and spinal cord at C3, C6, third thoracic segment (T3), T7, T12, first lumbar segment (L1) and L4. Following fixation, hooves were cut initially on an electrical band saw and three sites were sampled with razor strips for histology: through the junction of periople with coronary hoof at right angles to the periople; through the junction of coronary with laminar hoof perpendicular to laminae; and through distal laminar hoof (Dyce et al., 1987). Histological preparations of hoof included dermis, epithelium, stratum lamellatum, stratum medium and stratum tectorium; no hoof keratin softening agents were used.

Results (general): There were no obvious clinical effects attributable to Se in any treatment group fed Se with their feed during or after Se exposure. In particular there was no overt lameness, visual impairment nor hair loss. Although there was no significant difference in weight gain between treatment groups by ANOVA ($p<0.05$), controls gained slightly more than principals at 84, 105 and 120 days (Fig. 5). One high dose Semet steer (#14) developed a complete aversion to the treated diet on day 10 and was replaced in the experiment by a similar animal from a local herd (#80). The aversion exhibited by this steer (#14) was so severe that he completely refused hay for several days whether or not it was amended with Se and refused to eat anything fed in one of the key-controlled feeders for weeks. He was force fed by stomach tube for 5 days, after which he began to eat blue grass (lawn) and whole stem hay. As a separate experiment, he continued to receive his daily dose of Semet (0.8 mg/kg)

via gelatin capsule for the remainder of the feeding trial.

There were no statistical differences between treatment groups in any of the clinical chemical parameters measured (BUN, HBDH creatinine, CK, AP, ALT, bilirubin, glucose, albumin, protein) or the CBC nor did any of the principals deviate from historical normal ranges. In contrast to earlier reports (Franke and Potter, 1934; Draize and Beath, 1935) there was no evidence of anemia in the red blood cell count, hemoglobin or hematocrit. We have never identified anemia in either spontaneous or experimental selenosis, but it is sometimes cited as an chronic toxic effect of Se, especially in small carnivores (dogs, cats) and laboratory rodents. This discrepancy may reflect species differences in Se metabolism or differences in other dietary factors which interact with Se.

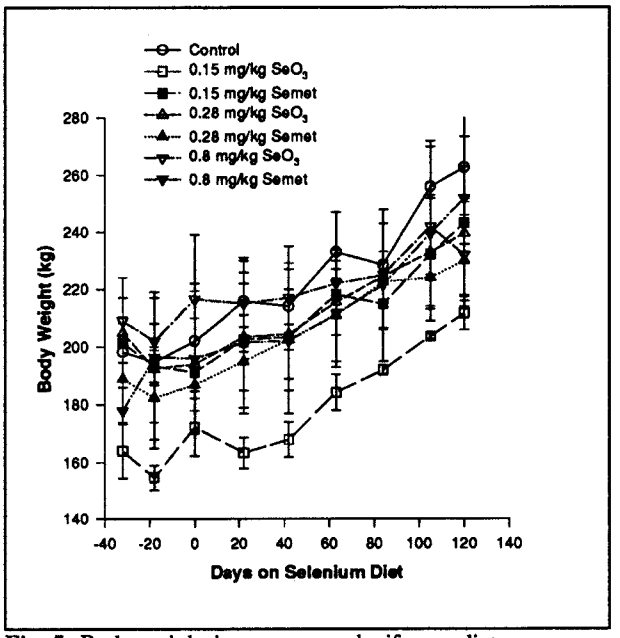

Fig. 5 Body weight in steers on seleniferous diets.

Glutathione peroxidase activity increased in both control and Se-treated steers throughout the feeding period (Fig. 6). The increase occurred earlier in the 0.15 and 0.28 mg/kg Se-treated groups than in controls or 0.8 mg/kg Se-treated steers, however, there was a great deal of variability in this parameter during the course of the experiment. Taken over the course of the feeding period, any increase in the Se-treated groups was no greater than the seasonal variation in controls. According to the theory prevailing at the time this experiment was conducted, GSHPx activity increases in tandem with dietary Se as it is increased from deficient concentrations then it plateaus sometime after adequate dietary Se status is achieved. Dietary Se in excess of nutritional requirements, especially toxic concentrations, should not result in increased GSHPx activity. We reasoned that the blood Se:GSHPx ratio might be used to recognize incipient selenosis, but the former hypothesis has been recently challenged. In this experiment all animals were either already at optimum GSHPx activity, or else other, seasonal factors were more influential. Interestingly, GSHPx in steer #14 (see below) decreased precipitously to "Se-deficient" levels shortly before he developed overt clinical signs of selenosis. We have seen a similar effect in field cases of alkali disease

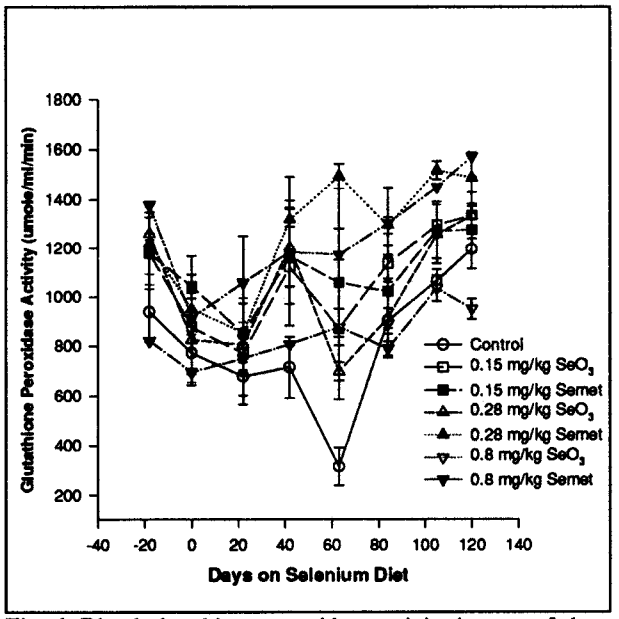

Fig. 6 Blood glutathione peroxidase activity in steers fed SeO_3^- or Semet.

in horses and cattle (Raisbeck and Hamar, unpublished). The Se:GSHPx relationship needs closer examination in appropriate target species to determine if it is diagnostically useful.

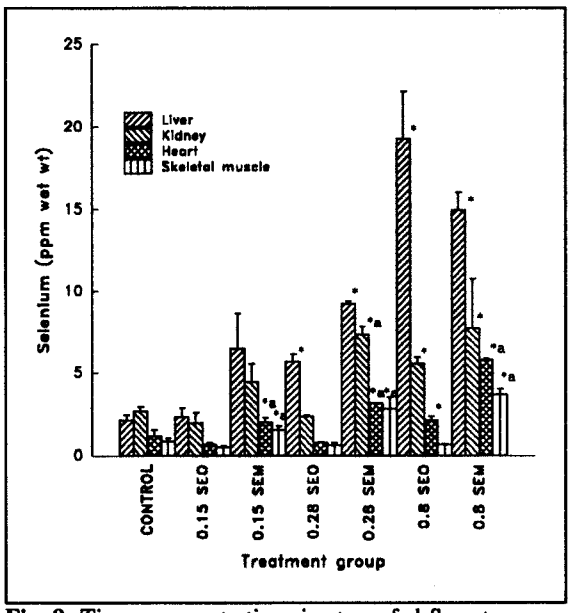

Fig. 8 Tissue concentrations in steers fed Semet or $NaSeO_3$ for 120 days.

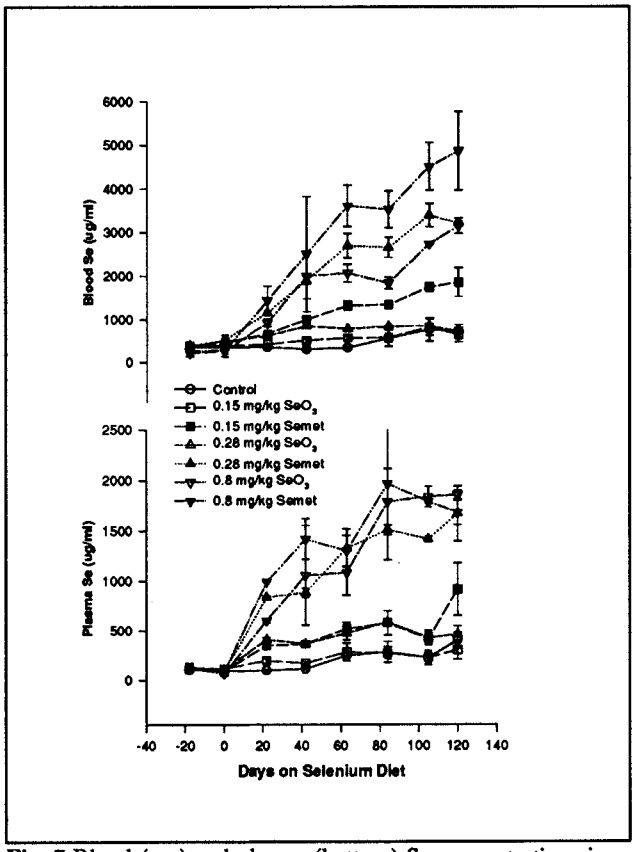

Fig. 7 Blood (top) and plasma (bottom) Se concentrations in steers fed $SeO_3^=$ or Semet. Note greater variability in plasma concentrations.

Blood and plasma Se concentrations increased from baseline values in steers fed either Se diet (Fig. 7). Plasma Se concentrations increased more rapidly and were generally more labile than whole blood Se. With the possible exception of the high $SeO_3^=$ group, blood concentrations appeared to plateau between 60 and 80 days. Dietary selenomethionine resulted in significantly higher blood concentrations than did corresponding dosages of $SeO_3^=$. *Post mortem* tissue Se concentrations, with the exception of liver, were also significantly greater in selenomethionine-fed steers than $SeO_3^=$ (Fig. 8). The latter differs from the situation in waterfowl where selenomethionine resulted in significantly greater accumulation in liver (Heinz et al., 1988).

Results (immunology): Evaluation of blood taken from steers in this experiment revealed no Se-related effects in any index of immune competence which relied on *in vitro* manipulation of immunocytes (ie. lymphocyte blastogenesis with phytohemagglutinin (PHA), lipopolysaccharide (LPS), concanavalin-A (Con-A), interleukin 2 (IL2) or IL4 production). Selenium exposure did not influence general measures of immune status such as total lymphocyte counts or total immunoglobulin (Ig) concentrations in circulating blood. However, there was a dose-related decrease in production of specific antibodies in response to a foreign protein challenge (OVA) administered while steers were on high-Se diets (Fig. 9). The last result is similar to that seen in rodents fed selenocystine, Na_2SeO_3, and/or Semet (Schamber, 1994; Schamber et al., 1995; Raisbeck, et al., 1997) and appeared to have a threshold of approximately 10 ppm dietary Se. The fact that only antibody response to an

antigen given while on seleniferous diets decreased suggests that Se somehow interferes with primary immune response rather than non-specifically destroying immune cells. Further work with more animals is needed to assign accurate threshold(s) to this effect, but, under field conditions, Se induced susceptibility to infectious diseases could indirectly result in losses more extensive than overt toxicity.

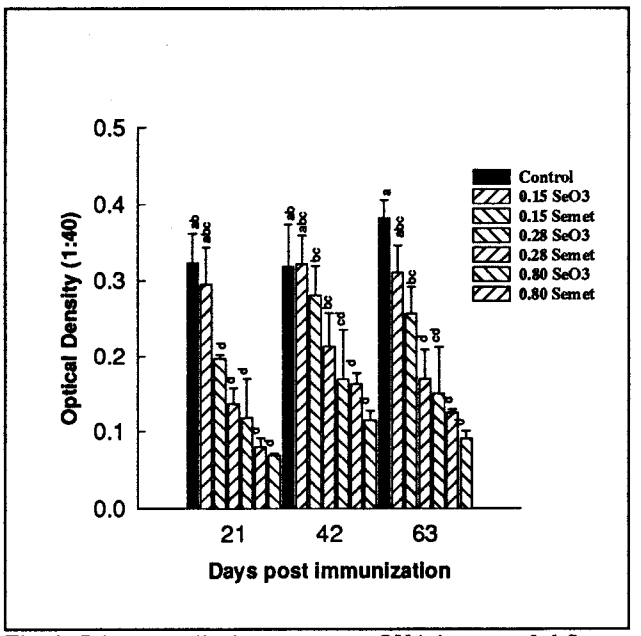

Fig. 9 Primary antibody response to OVA in steers fed Se. Similar letters denote statistical similarity.

Results (pathology): At *post mortem*, one 0.8 mg/kg Semet steer (#15) exhibited circumferential ridges around the hoof parallel and distal to the coronary band. No other gross lesions were identified in steers fed Se-treated hay. No statistically significant differences were noted in organ weight:brain weight ratios between control and experimental animals. Histologic lesions were confined to hooves of both 0.8 mg/kg Semet steers, one 0.8 mg/kg $SeO_3^=$ steer and one 0.28 mg/kg Semet steer. We examined the central nervous system with particular care for any lesions which might account for the syndrome known as "blind staggers". There were none. Lesions which occurred in both control and Se-fed groups were interpreted as being incidental (ie. unrelated to Se).

The histologic lesions in hooves of affected steers have been described in detail elsewhere (O'Toole and Raisbeck, 1995) and are similar to those seen in field cases we have examined (Raisbeck and O'Toole, 1997). Briefly, these involved the keratin forming tissues of the coronary band and epidermal laminae and were characterized by hyperplasia, acanthosis and parakeratosis especially near the tips of affected laminae. In affected areas, the germinal epithelium lost its normal regular columnar structure and became very disorganized and attenuated. Similar, albeit milder, multifocal lesions occurred in the coronary epidermis. As a result, the hoof wall, which in normal animals derives its strength from numerous, regularly spaced small (15 um) tubules, contained fewer but larger (75 um - 250 um), thin walled tubules filled with cellular debris. Decreased tubule numbers and wall thickness are probably responsible for the structural weakness which eventually results in hoof lesions.

Results (steer #14): Steer #14, which received 0.8 mg Semet/kg bw daily via gelatin capsule was the only steer in this study to develop overt clinical selenosis during the feeding trial. During the initial period of feed refusal he lost 46 kg, approximately 25% of his body weight. He gradually regained his baseline body weight over the subsequent 6 weeks, but never caught up with his peers. Failure to gain weight normally may be a direct toxic effect of Se or may merely have been a sequela to the severe weight loss he suffered while refusing feed. Blood and plasma Se concentrations were greater than steers which received a similar dose of Semet mixed with their daily ration. Although it is possible that this single steer may have been unusually susceptible, it appears that Semet given as a single daily bolus is more

bioavailable, possibly as a result of greater rumen bypass, than when consumed over a 24 hr period with feed. This fact should be taken into account when designing or interpreting Se feeding studies in ruminants.

After receiving Semet for 96 days, steer #14 exhibited mild lameness, slight swelling and erythema just proximal to the coronary band in both front legs. These signs subsided for a few days then, ten days after the initial signs a hairline crack appeared parallel to and 0.5 cm distal to the coronary band and the steer became markedly lame. Hoof separation and lameness both increased in severity until, by 120 days, he refused to walk to feed and water and had to be hand fed.

At *post mortem*, new hoof growth was found underlying the separated horny tissue. Histopathologic examination of the hooves of steer #14 revealed lesions qualitatively similar to those described above, but markedly more severe. After about 100 days on Semet this steer's tail switch disappeared, leaving the distal 6 cm of the tail denuded. Histologic examination of skin from this area revealed atrophy in a high proportion (~75%) of primary hair follicles. Primary follicles from an unaffected, more proximal portion of the tail were only about 10% atrophic. Secondary follicles were unaffected. Samples from other sites on this animal were unaffected as were samples from all other animals (O'Toole and Raisbeck, 1995).

Lay summary - steer feeding experiment(s):

1. Chronic exposure to diets containing more than 10 ppm Se resulted in subclinical but measurable damage to the immune and integumentary systems of yearling steers over the course of a period typical of a Summer grazing season in Wyoming. The integumentary lesions identified in our laboratories (O'Toole and Raisbeck, 1995; O'Toole and Raisbeck, 1997; Raisbeck and O'Toole, 1997) are sufficiently unique to be a useful adjunct in identifying selenosis under field conditions. Immunotoxic effects, while very difficult to evaluate under field conditions, have potentially greater impact than those previously reported for Se.

2. These effects, when they occurred, were associated with blood concentrations greater than 3.0 ppm, liver greater than 9.0 ppm and kidney greater than 5.0 ppm. However, Se tissue concentrations *per se* were not an accurate predictor of Se-induced effects in these animals and we have identified clinical selenosis in field cases with smaller concentrations. Thus identification of selenosis on reclaimed mine lands must rest on all three legs of the triad of clinical signs, pathology and chemical analysis rather than chemistry alone.

3. Selenium from Semet accumulated to a greater degree than did $SeO_3^=$ in all tissues examined with the possible exception of liver. Liver from the 0.8 mg/kg $SeO_3^=$ dose group was non-significantly higher than the similar Semet group. Semet seems to be marginally more chronically toxic in herbivores than $SeO_3^=$, possibly as a result of this accumulation.

4. Given available funding, this study was necessarily limited in statistical power, and thus not designed to identify an absolute "toxic level" *per se*. Nonetheless the results of this particular experiment support the concept of a 5 ppm vegetation Se cutoff for grazing ruminants.

Antelope experiment

After the intensive feeding trial in steers to describe the clinical, toxicologic and pathologic syndrome of chronic selenosis, our original proposal called for validating the basic experiment in a second species of Great Plains herbivore. We were unable to obtain sufficient purified Semet for the originally proposed antelope study and therefore substituted seleniferous native grass hay as our source of Se in the experimental diets.

Model: Nine healthy male pronghorn antelope aged 6 mo to 8 yr were obtained from the Colorado Division of Wildlife Foothills Wildlife Research Facility and maintained in outdoor pens at the Sybille Wildlife Research Station from October to through May. Animals either were born to captive pronghorn in the Colorado facility or raised from wild-caught perinatal fawns. Each pen was provided with a heated waterer (<50 ppb Se), free-choice NaCl and a three-sided shed for shelter.

After acclimation to a ground (~1 cm) grass hay-alfalfa diet (basal diet; 0.3 to 0.5 ppm dm Se) for 1 mo, animals were ranked by weight and, from that ranking, randomly assigned to either a control (nos. 1 to 4) or a experimental group (nos. 5 to 9). These groups were then subdivided by age (fawns and adults) between four pens, and fed *ad lib* in wooden feed bunks. Feed consumption was monitored by weighing unconsumed feed. Experimental animals were fed a diet of seleniferous native grass hay (23 to 25 ppm Se) ground 60:40 with alfalfa (0.3 to 0.5 ppm Se). The Se concentration of the experimental diet was analyzed after each batch was ground and found to vary between 14 and 16 ppm Se (dm). Control pronghorn remained on the basal ration. From late January (49 days on treatment) until the experiment was terminated, each animal also was given 200 g of either a corn-oats-barley mix or alfalfa pellets (0.3 to 0.5 ppm Se) per day.

Endpoints: Each animal was examined daily for any evidence of ill-health and weighed weekly. On day 28 each pronghorn was given a single subcutaneous injection (2 ml) of OVA, 20 mg/ml in potassium alum adjuvant (Proom, 1943). Blood was drawn into evacuated containers every 28 days by jugular venipuncture for Se analysis, clinical chemistry (same analyses as above), complete blood count and immunologic analysis (as for steers). Samples for Se and antibody analysis were frozen -20C until analyzed. Blood counts were performed within 24 hr and clinical chemistries within 8 hr of collection on fresh blood and serum. After 164 days on Se hay, each pronghorn was anesthetized with xylazine[12], killed by electrocution and subjected to a complete necropsy and histopathologic evaluation similar to that used with the steers.

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[12] Rompun, Haver Scientific, Shawnee, Kansas, USA

Results: Although antelope initially ate the ground grass/alfalfa diet readily, feed refusal by both the control and especially the experimental diets was a recurring problem from day 30 until the end of the study. Poor feed consumption, together with the extremely cold weather necessitated supplementation with a small amount of grain as noted above. We thus aren't certain whether poor weight gain seen in the experimental fawns was directly attributable to Se or an indirect result of poor palatability and feed aversion. The latter seems more likely as gain decreased at about the same time as feed consumption (Fig. 10). Due to the small number of animals and the loss of a control animal to an iatrogenic infection, statistical analysis was not attempted. Clinical pathology and chemistry did not reveal any apparent differences between groups, nor deviations from historical normals, with the exception of GSHPx which was elevated in Se-fed animals (Fig. 11). Blood and tissue Se concentrations in Se-fed antelope were similar to the bovine 0.15 mg/kg Semet and 0.28 mg/kg $SeO_3^=$ groups. Histopathologic evaluation of tissues, including a detailed examination of hooves did not reveal any evidence of Se-induced effects. There were no consistent trends in the *in vitro* immunocompetence assays attributable to the high Se diet. However we must note that intradose variability in the latter parameters was large, probably due to collecting and transporting samples in subzero weather and the excitable, easily stressed nature of this species. On the other hand primary antibody response to OVA was significantly less in animals on the high Se hay diet than in controls.

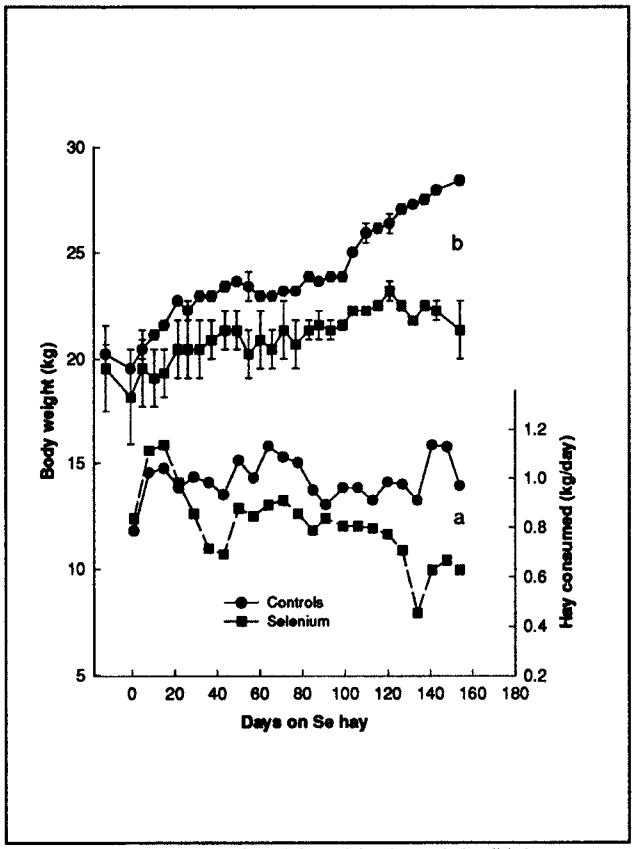

Fig. 10 Feed consumption (a) and body weight (b) in antelope fawns fed a seleniferous diet.

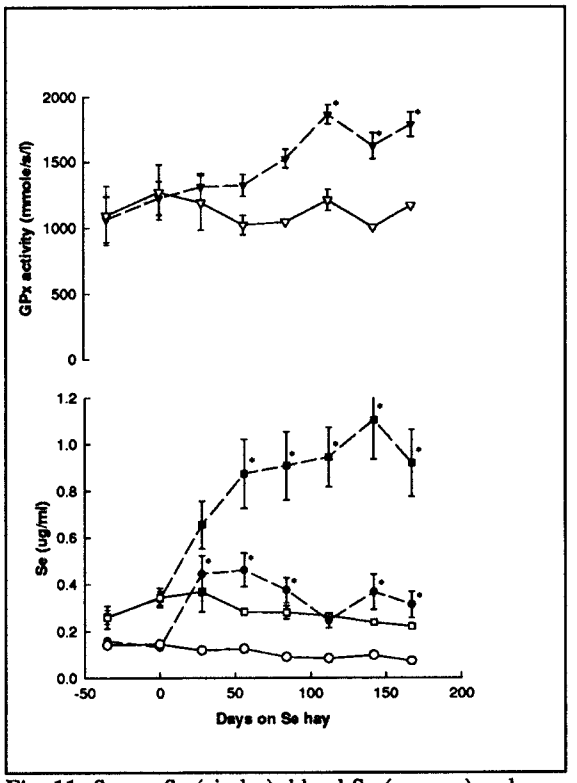

Fig. 11 Serum Se (circles), blood Se (squares) and GSHPx (triangles) in antelope fed control (open figure) or seleniferous (closed) diets.

Lay summary - antelope experiment

It has been suggested that antelope, being native to the Great Plains, are intrinsically more resistant to selenosis than domestic livestock. The results of this experiment, unfortunately, neither completely prove nor disprove that contention.

1. Blood and tissue concentrations in antelope fed a 15 ppm diet were similar to cattle dosed with either 0.15 mg Se/kg bw as Semet or 0.28 mg Se/kg bw as $SeO_3^=$, ie. the bioavailability of Se was slightly less than or equivalent to cattle. This conclusion is confounded by two factors. In order to maintain antelope through winter weather we were forced to feed concentrates. This may have resulted in a decrease in hay consumption. Secondly, we weren't able to characterize the species of Se in the hay we used. This particular lot was harvested very late in the year and had weathered badly before we obtained it. The increased GSHPx in the Se-fed group suggests that, unlike steers, antelope were making use of the additional dietary Se.

2. Primary antibody response, which was decreased by Se in cattle, was also decreased in antelope. This lends credence to our conclusion that immunotoxicity is a potential real toxic effect of Se in grazing species.

3. There were none of the integumentary lesions seen in cattle. This is not, however, a completely valid comparison between experiments as the maximum dosage we could achieve with seleniferous hay was less than that at which lesions occurred in all but one of the steers. A single steer was maintained on undiluted seleniferous hay from the same source of hay as used for the antelope for more than a year before developing alkali disease. Hoof lesions, albeit incompletely characterized, have been reported in wild antelope in the past (Post, 1958). It does seem safe to say, however, that antelope are no more susceptible to selenosis than cattle.

Grazing Validation Study

The final phase of this project was to be validation of experimental results with natural exposure. There are two general methods by which to develop natural exposure data: 1) feeding diets derived from naturally seleniferous hay; or 2) pasturing animals on reclaimed mine sites with varying forage selenium concentrations. We opted for the latter in the summer of 1995 as it seemed more representative of the real world situation in the Powder River basin.

Model: Cattle were again used as the test species. For economic reasons we borrowed cattle from cooperators at most sites. Five potential grazing sites were selected on the basis of conversations with DEQ and mine personnel to represent the likely spectrum of Se exposure in the Powder River basin. Four sites were reclaimed pastures at active mines (A,B,C,F). Two (C, F) were in the northern Powder River Basin near Gillette, WY the other two (A, B) were further south, near Wright, WY. The owner of two of the reclaimed sites (C&F) dropped out of the study prior to getting underway. Twelve heifers were chosen at random from a larger group grazing site A. Site B received 12 steers from the Wyoming Hereford

Fund herd. The fifth (E), a ranch with a history of alkali disease, served as a positive control. The latter was necessitated by our inability to find a cooperating mine with known toxic vegetation. Twenty heifers were randomly selected from a group of approximately 76 cattle on this pasture. Twelve steers, pastured at the USDA High Plains Grasslands Research Station in Cheyenne, served as a negative control (site D). All cattle received routine preventive health treatments according to their owner's usual practices. Each site provided livestock quality water and plain white salt (NaCl) *ad lib*. Water quality was verified by analysis at the Wyoming State Veterinary Laboratory.

Endpoints: After baseline blood samples were taken, cattle were released on each of the sites. In order to estimate Se intake, dietary botanical composition was determined by visually observation eating habits for four hours morning and evening at monthly or more frequent intervals. Representative 500 g samples of grazed vegetation were collected onto dry ice for transport to the laboratory. Forage samples were frozen at -70 C to minimize Se loss, then lyophilized, ground and homogenized before Se analysis. Forage, blood and serum were analyzed as previously described. Se concentrations were averaged by plant species, collection date and site (species mean), then Se intake (dietary mean) was calculated from species means on the basis of the percentage each plant species contributed to the diet. Each animal was immunized with OVA as before and immune competence was evaluated by quantifying PAR in blood and by skin testing delayed type hypersensitivity to an OVA challenge.

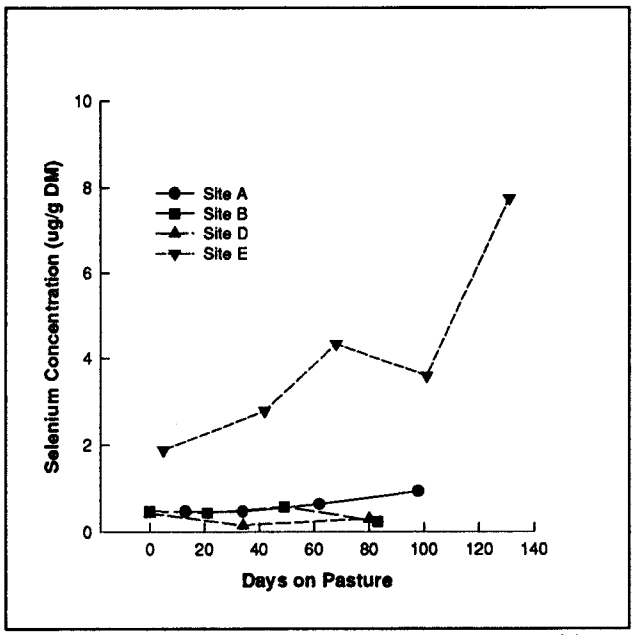

Fig. 12 Selenium concentration of vegetation consumed by cattle on reclaimed mine lands and seleniferous pastures.

Results: The summer of 1995 was extremely wet and forage conditions were unusually lush at all sites. Earlier investigators reported that forage Se concentrations peak during the late Spring (Dinkel et al., 1963). In our study forage concentrations peaked later in the growing season, typically August. This discrepancy may be due the different geology and forage ecology of northeastern Wyoming as compared to South Dakota. It may also be due to the fact that samples in this study were deliberately biased toward what cattle consumed rather than randomized samples of the biomass. None of the grazing sites (even site E) contained any indicator (obligate accumulator) species and all contained a few facultative accumulator specimens. Sites A, B and E had individual forage samples which exceeded 2.0 ppm, but only on site E did species mean concentrations exceed 2.0 ppm. On the latter site, several species of grass had mean concentrations that approached 10 ppm, and individual samples exceeded 20 ppm. Sample to sample variability was also greatest at site E. The highest *dietary* mean Se concentrations at any sampling interval were less than 1.0 ppm at all sites except E which reached nearly 8.0 ppm (Fig. 12). Blood Se concentrations in cattle on all sites except E remained below 0.5 ppm, which is typical of cattle in northeastern Wyoming.

Mean blood Se peaked at slightly more than 2.0 ppm at site E and showed much greater variability than in the other groups (Fig. 13). None of the cattle grazing any of these sites showed any clinical evidence of health problems. Results of immunologic tests were inconclusive, apparently as a result of reagent failure.

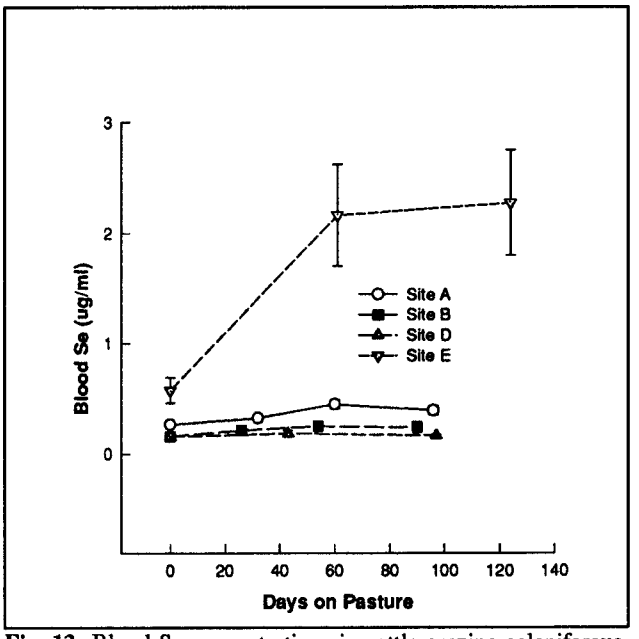

Fig. 13 Blood Se concentrations in cattle grazing seleniferous pastures.

Hay feeding study

Model: After failing to generate a usable range of dietary Se concentrations, tissue concentrations and health effects markers from the pasture experiments of 1995, we undertook a final feeding experiment with seleniferous hay in steer calves. Twelve yearling steers were obtained from the Wyoming Hereford Fund herd, divided into 3 groups and housed in concrete pens at the UW Department of Veterinary Sciences Research facility. Prior to this study, they were pastured on non-seleniferous pasture at the High Plains Grasslands Research Station in Cheyenne, WY. Water and white salt were provided *ad lib*. Each group was fed a ground hay ration prepared from seleniferous and non-seleniferous native grass hay and containing either 3.33, 5 or 6.66 ppm Se for 160 days. Diets were analyzed and balanced with corn and cottonseed meal to be isocaloric and isonitrogenous. Steers were observed daily for any signs of adverse health effects and bled and weighed every 21 days.

Results: Blood Se concentrations increased in a dose-related manner in all groups (Fig. 14), and plateaued at 0.5, 0.8 and 1.4 ppm in the low, mid and high Se groups respectively. There were no significant differences in rate of gain between groups (Fig. 15), nor did any of the steers exhibit any clinical evidence of disease during the 6 months they were on seleniferous hay. Assuming blood concentrations plateaued by the end of the feeding period, regressing blood concentration vs dietary concentration yielded [blood]=0.2701*[hay]-0.449. A similar regression of maximum dietary forage concentrations from the previous study yields a similar slope but smaller constant ([blood]=0.2816*[grass]+0.0871).

Lay summary - Natural Se feeding studies:

1. The primary goal of the last two feeding studies was to validate results of the initial, controlled experiment with "natural" dietary Se under conditions similar to Powder River Basin grasslands. The unusual rainfall and moderate Se concentrations in most pastures precludes extensive correlation. That is, because none of the grazing study animals were exposed to toxic (as evidenced by intoxication) dietary concentrations, we cannot draw definitive conclusions about the nature of spontaneous selenosis on reclaimed mine lands. Nonetheless, the fact that none of the cattle in this experiment, including site E which had a total of 76 cattle, developed any signs of selenosis

supports the premise of a dietary threshold between 5 - 10 ppm Se from natural (forage) sources.

2. A second, subsidiary goal of this last set of experiments was to derive a mathematical (toxicokinetic) relationship between forage Se and steady-state blood Se concentrations under conditions typical of the Powder River Basin. Such would facilitate comparison between experimental studies and naturally occurring disease. We found that, at *moderate exposures*, blood Se increased more or less linearly with dietary concentration. The slope (ie. the rate at which increased dietary Se was reflected in increased blood concentrations) was similar for hay, pasture grass, and for purified Semet (Fig. 16). This suggests that forage Se, while not identical to Semet is closer than to Semet than inorganic Se. At higher exposures, the tissue concentrations achieved by inorganic Se are closer to those achieved by Semet, possibly because of high tissue concentrations of inorganic Se poison its elimination mechanism (Blodgett and Bevill, 1987).

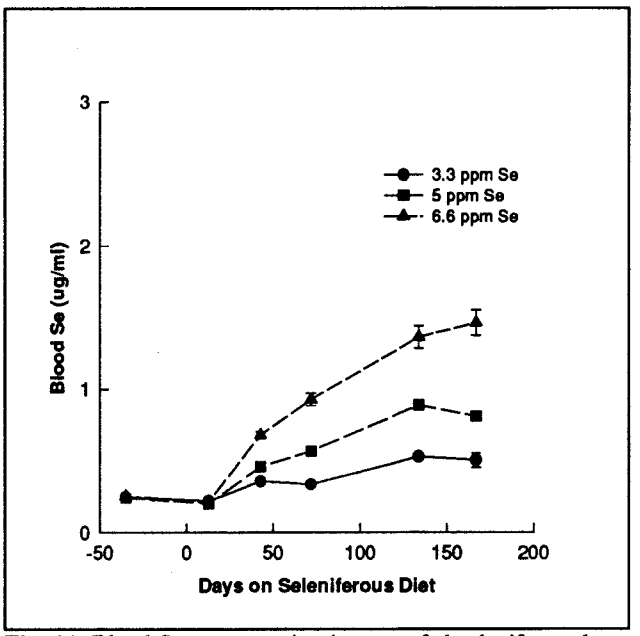

Fig. 14 Blood Se concentration in steers fed seleniferous hay diets.

Project Summary

"Toxicologic Evaluation ..." was undertaken to qualitatively describe the pathophysiology of chronic selenosis in ruminants, a condition which constitutes the major starting point of many reclamation decisions and regulations. We used two species of primary consumer which are both common and economically important in the Powder River mining area and focused on endpoints which would be useful in field studies of the rock-soil-plant-herbivore Se cycle. Economic limitations led us to exclude horses and large scale reproductive experiments. We have ongoing (eg. Gamble et al., 1997) and planned (Galey and Raisbeck, 1997) projects with non-AML funding which will hopefully help fill some of these gaps.

We were unable to detect adverse health effects in any animals fed less than 10 ppm Se. These experiments were not designed specifically to derive a no-effect level (such would require many more animals and cost proportionately more) and, as such, lacked sufficient statistical power to be conclusive. In large populations there will probably be sensitive individuals. There are also many environmental factors such as nutrition or concurrent disease which may potentiate selenosis. Nonetheless: 1) in none of the experiments or field studies undertaken since 1989 have we been able to convincingly link chronic selenosis in ruminants to forage Se concentrations less than 5-10 ppm and 2) many of the animals we've followed have apparently tolerated this same range.

The most distinctive *chronic* toxic effect of Se is dyskeratosis manifested as a particular pattern of hair loss and hoof deformities. A technical description suitable for use by trained pathologists is available in the open literature (O'Toole and Raisbeck, 1995, 1997; Raisbeck and O'Toole, 1997). An equally- or possibly more-sensitive toxic effect is inhibition of PAR. Unfortunately, this condition may be produced by many other toxicants, infectious agents and nutritional deficiencies and is thus much less specific than pathology. It is, however, an easily evaluated endpoint for experimental use if appropriate controls are used. It also raises the question of the health effects of concurrent exposure to Se and infectious diseases.

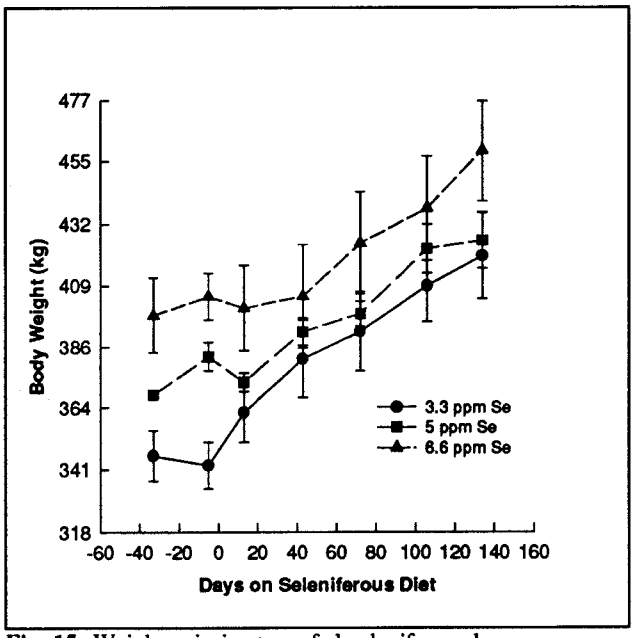

Fig. 15 Weight gain in steers fed seleniferous hay.

Several observations from non-AML experiments and field investigations undertaken during this same period bear mentioning. Our initial impression of the 5 ppm "magic number", based on 3 years of field observations, was that it was excessively conservative. At the same time some advocated interpreting this "toxic level" to mean the presence of *any* vegetation greater than 5 ppm Se rendered a whole pasture hazardous and were pressing for even lower reclamation limits. After several hundred hours of observing cattle graze we are convinced that seleniferous vegetation has to be widely and fairly uniformly distributed to pose much of a hazard to well managed cattle (antelope seem to be even pickier) and that pasture evaluation should focus on non-accumulator plant species, or (preferably) biomonitors such as grazing animals which integrate all of the variables involved in Se bioavailability.

Depending upon where one looks, the older literature suggests that horses may be more, less, or equally sensitive to forage Se. To the best of our knowledge there have never been any controlled studies of the potency of naturally seleniferous forage in this species. We have, however, investigated a case in North Dakota in which alkali disease occurred in horses fed 4 ppm Se grass hay, and several in Wyoming in which horses were alkalied on the same pasture as unaffected cattle.

Many of the popular summaries of early selenosis research describe infertility as a prevalent effect in livestock (Dinkel, 1963; Rosenfeld and Beath, 1964; Olson, 1978; Beath, 1982). More recent experimental studies in lab animals, sheep and non-human primates have either not been able to produce such effects or produced them only at doses which produced overt intoxication (Willhite, 1993; Panter et al., 1995). Veterinarians involved with earlier Wyoming selenosis investigations hypothesized that reported reproductive failures were secondary to starvation resulting from the crippling effects of selenosis (J. O. Tucker, personal communication). To date our field investigations of ranches with histories of both alkali disease and reproductive failure revealed animals which tissue analysis indicated were copper deficient as well as extremely high in Se. So far we haven't investigated reproductive

complaints associated with elevated Se without concurrent hypocupremia. Intervention with copper supplementation in one instance increased pregnancy from 80% - 100%. Herds grazing high Se pastures without concurrent trace element deficiencies seem to be limited to more conventional alkali disease. This situation, while outside the scope of the AML program deserves much more extensive investigation.

Research funded all or in part by this project has characterized chronic selenosis in ruminants. The information disseminated in the resulting thesis and research publications provides a solid foundation for investigation of naturally occurring selenosis in Wyoming.

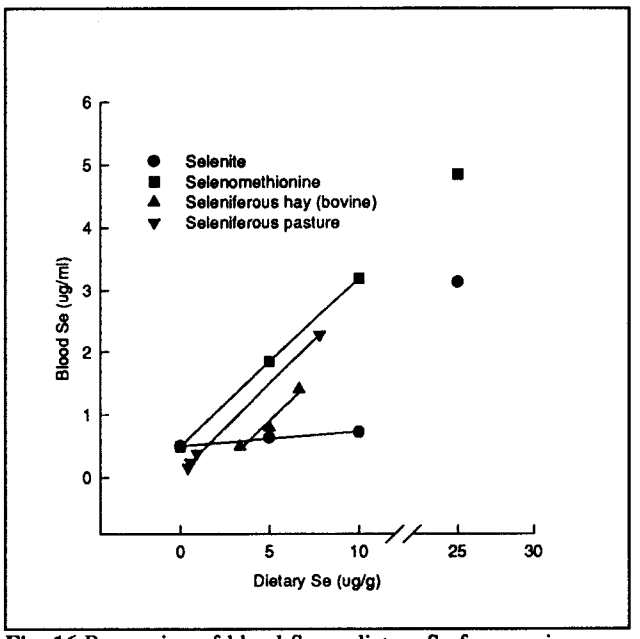

Fig. 16 Regression of blood Se vs dietary Se from various sources.

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University of Wyoming Department of Veterinary Sciences

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RE-EVALUATION OF SELENIUM TOXICITY IN GRAZING MAMMALS[1]

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Abstract: The potential uptake and concentration of selenium by vegetation grown on abandoned coal mine lands has been the focus of many restrictions governing the reclamation of such sites. While there is little question that selenium is very toxic, recent research suggests that much of what has been anecdotally attributed to selenium in the past, is, in fact, due to other environmental factors. This presentation will summarize research undertaken by the authors during the last 6 years to more clearly define the effects of selenosis on herbivores in Wyoming.

Additional Key Words: selenosis, poisoning, antelope, cattle

Introduction

Selenium (Se) is a group VI element that is chemically very similar to sulfur. In North America, it is reportedly most abundant in Cretaceous shales and glacial drift of the Great Plains. Concentration by leaching, evaporation and/or plant uptake may result in forage concentrations which are potentially toxic to grazing mammals. The fact that these natural processes may be exacerbated by anthropogenic activities such as surface mining and irrigation resulted in increased control of such activities during the 1980's to prevent future Se toxicity to livestock and wildlife.

It has become fashionable to begin any paper on the toxic effects of Se by citing historical documents such as Marco Polo's journal as early reports of selenosis (Trelease and Beath, 1949a; Harris, 1991; James et al., 1990). In point of fact, however, the first conclusive evidence of naturally-occurring Se toxicity (selenosis) in mammals was contained in reports from the Wyoming and South Dakota Agricultural Experiment Stations during the 1930's (Beath et al., 1934; Franke, 1934a; Franke, 1934b; Franke & Potter, 1934). During the subsequent 2 decades, a model of selenosis emerged from experiments and field studies undertaken by these laboratories and the USDA Bureau of Animal Industries. According to this model, ingestion of toxic amounts of Se, either as purified inorganic salts or as accumulated in range plants, resulted in one of several distinct pathophysiologic entities: acute selenosis, experimental chronic selenosis, "blind staggers" and "alkali disease" (Trelease and Beath, 1949b; Rosenfeld and Beath, 1964).

The discovery in the 1950's that Se was, in addition to being highly toxic, an essential trace element (Schwartz and Foltz, 1957) produced a marked shift in emphasis in biomedical Se research. Relatively little original research into the toxicity of Se was conducted during the 1960's and 1970's; the dogma of the 1930's and 1940's became enshrined in the scientific literature by repetitive citation. Thus, it is not surprising that the popular press and, more importantly, regulatory personnel struggling with reclamation standards accepted this paradigm uncritically.

Acute selenosis is of limited relevance to range animals for two reasons. First, acute selenosis requires quantities of Se that animals are very unlikely to ingest voluntarily under range conditions. For example, an acutely lethal oral dose of Na_2SeO_4 for a 400 kg cow contains about 4 gm Se. Assuming that this dose was *evenly* distributed throughout a single day's forage intake, the final Se concentration would be about 500 ppm. While the so-called "accumulator" plant species may accumulate this much Se or more, the chemical form is such as to render the plant extremely unpalatable. As noted by Beath (1920), "when cattle were placed

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in enclosures on *astragalus* they would not touch the plant at all and after a few days could not be kept inside the fence". Second, many accumulator species such as two-grooved milkvetch (*A. bisulcatus*) contain other, more significant toxins such as swainsonine, which render the theoretical toxicity of any Se present moot.

The extent of selenosis in Wyoming

The popular perception that "Extensive livestock losses have occurred and continue today" (Boone, 1986) isn't consistent with the experience of animal health researchers and stockmen in the western United States. For example, a review of the records of the Wyoming State Veterinary Laboratory from its inception in 1947 through 1988 revealed no confirmed cases of selenosis (L.W. Woodard, 1988). Reportedly, Se intoxication in livestock is most commonly the result of over-supplementation rather than natural sources (Edmonson et al., 1993). Similarly, a prospective survey of selenosis in domestic livestock conducted by the University of Wyoming from 1988 through 1991 found only isolated instances of the disease (Raisbeck, et al, 1993).

In the latter survey, which is still ongoing, cases of suspected selenosis in livestock were solicited from veterinarians and producers by announcements in the Wyoming Veterinary Medical Association newsletter and in various statewide livestock publications. Originally, submissions were restricted to cases of illness or death in which one or more clinical signs were similar to those reported in the older literature (Table 1), and for which there was adequate clinical history to permit correlation between the disease process and environmental Se concentrations.

Table 1: Criteria for inclusion of cases in original prospective survey of selenosis in Wyoming.

History of possible exposure to seleniferous plants

Availability of appropriate tissue samples for diagnosis

Clinical signs

Neurological
- Blindness
- Head pressing
- Circling
- Ataxia
- Tremors
- Convulsions

Epithelial
- Hair loss, especially of mane or tail
- Lameness, especially if accompanied by hoof deformities

Sudden death

Reproductive
- Poor conception rates not related to other causes
- Male infertility

The response to this original solicitation was very limited. Thus, in early 1990, the survey was widened to include any incident of livestock disease that the attending veterinarian or livestock owner believed might be associated with elevated dietary Se from any source. As of June, 1994, our lab had investigated 116 accessions[3]. Of these, six equine and two bovine accessions were attributed to Se on the basis of tissue concentrations and elimination of other possible etiologies. Two additional wild bird accessions which had extremely high tissue Se concentrations were suspect, but were too decomposed to permit any further diagnostic workup.

The clinical signs observed or reported in confirmed cases were similar to those described as the "alkali disease" form of chronic selenosis in the older literature (Trelease and Beath, 1949a). Typically, 1 - 2 months after exposure to the suspect feedstuffs, owners report noting alopecia of the mane and tail. Soft tissue swelling in the region of the coronet and/or a very fine, horizontal crack in the surface of the hoof just distal to the coronet are also noted at this time or soon thereafter. Lameness at this stage is variable. The hoof lesion and attendant lameness

[3] An accession is a single incident of a disease occurrence reported to our laboratory. It might represent a single pet horse, or an outbreak in a herd of cattle.

progressively worsen over a period of several months until, finally, the toe separates from the hoof and is lost. None of the cases we have investigated thus far exhibited any evidence of other organ (e.g. liver) involvement. No affected animals have died. It is, however, easy to see how death from starvation, arthritis, and/or many of the other conditions attributed to alkali disease might have resulted from the severe lameness if animals had been left to fend for themselves under range conditions.

Twenty-three accessions met the clinical criteria of the condition described as "blind staggers" (Draize and Beath, 1935; Trelease and Beath, 1949b). Of these, 10 were diagnosed as lead poisoning on the basis of tissue lead concentrations, eight were histopathologically diagnosed as polioencephalomalacia and the remainder attributed to various pathogenic infections. Only one had elevated tissue Se concentrations, which were judged to be diagnostically insignificant given the extremely high lead concentrations.

Experimental studies: cattle

Much of the experimental data regarding selenosis in large domestic mammals is derived from exposure to inorganic salts of Se such as Na_2SeO_4 (Maag et al., 1960; Miller and Williams, 1940; Olson and Embry, 1973). However, the predominant forms of Se in forage plant species are the seleno analogs of sulfur-containing amino acids, especially selenomethionine (Olson, 1977). Published data about the physiological effects of this form of Se are variable; however, there is evidence in trout (Hamilton and Buhl, 1990), waterfowl (Heinz et al., 1988; Fairbrother and Fowles, 1990) and laboratory mammals (Waschulewski and Sunde, 1988) that the chronic toxicity of selenomethionine differs both quantitatively and qualitatively from that of inorganic Se salts. A similar comparison in grazing (ruminant) species is thus very relevant to mine reclamation in Wyoming.

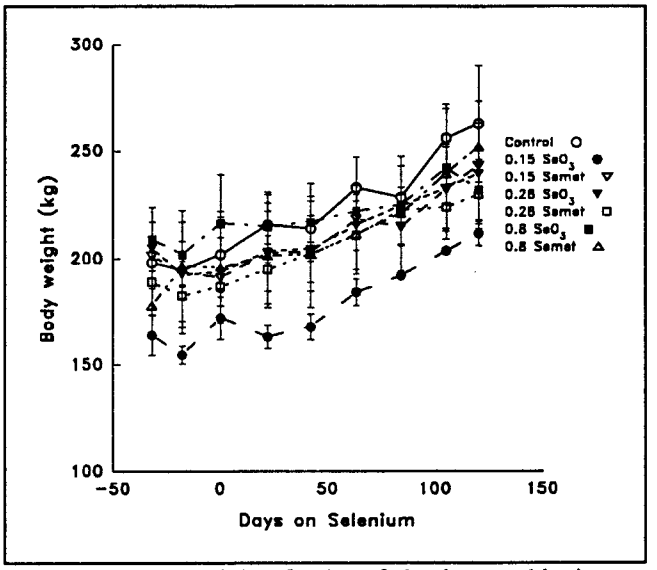

Figure 1: Body weight of calves fed selenomethionine or selenite at 0.15 mg Se/kg BW, 0.28 mg Se/kg BW or 0.8 mg Se/kg BW. Selenium was fed from day "0" through day 120. Each point represents mean ± standard error.

We undertook such a study with support from the Abandoned Coal Mine Land Research Project and the UW Agricultural Experiment Station during 1992-1993. Yearling steer calves obtained from the Medicine Bow area were fed a Se-adequate basal ration of grass hay typical of dryland pastures in the state. After a 45-day acclimation period, during which they were de-wormed and vaccinated for diseases common in Wyoming, steers were randomly assigned to either a "high", "medium" or "low" selenomethionine or $SeO_3^=$ group. Dosage rates (0.15, 0.28 or 0.8 mg Se/kg BW) were calculated to approximate 5, 10, or 30 ppm dietary Se under range conditions. Doses were prepared daily for each animal and thoroughly mixed with that day's ration of chopped hay. Rations for each animal were adjusted weekly to ensure that the entire dose was consumed. Control steers remained on the basal diet. Periodic blood samples were taken for Se, immunology and clinical pathology. After 120 days on Se, each steer was humanely killed and subjected to a complete necropsy with a detailed histopathologic review (Appendix 1).

Both experimental and control calves appeared clinically healthy throughout the duration of the experiment. All gained weight throughout the 120 day exposure to Se. Although there was no statistically significant difference between treatment groups by ANOVA ($P<0.05$), controls gained more than the high $SeO_3^=$ group at 84, 105 and 120 days (Figure 1). There were no variations attributable to Se in any of the clinical pathologic parameters measured (blood urea nitrogen, serum creatinine, serum creatinine kinase, serum alkaline phosphatase, serum aspartate aminotransferase, serum bilirubin, serum glucose, serum albumin, serum

total protein, red blood cell count, white blood cell count, hemoglobin, hematocrit, or differential white cell count). Of particular interest, and in contradiction to early reports (Franke and Potter, 1934; Draize and Beath, 1935), there were no changes in erythrocytic parameters that indicate anemia.

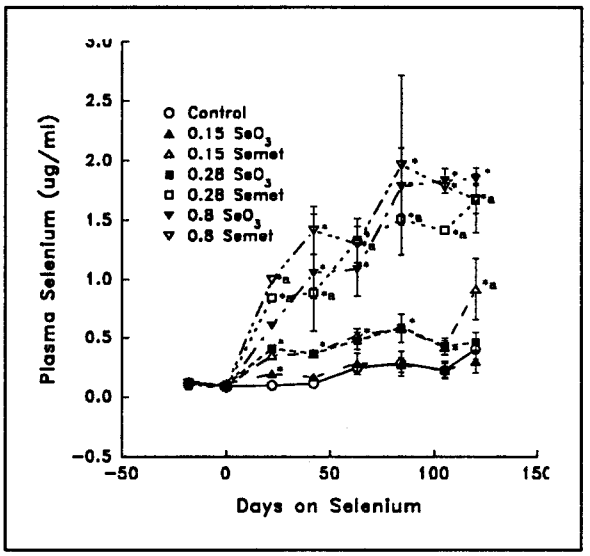

Figure 3: Plasma Se concentration (mean ± SE) in calves fed selenomethionine or selenite at 0.15, 0.28 or 0.8 mg Se/kg BW. "*" indicates significantly greater than controls, "a" greater than corresponding dose of SeO_3.

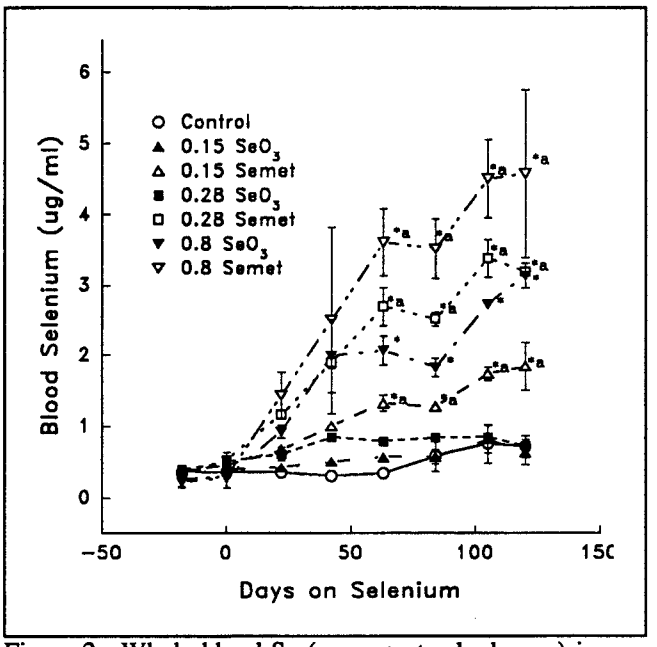

Figure 2: Whole blood Se (mean ± standard error) in calves fed selenomethionine or selenite. "*" indicates significantly greater than controls, "a" greater than corresponding dose of SeO_3.

Not surprisingly, blood and plasma Se concentrations increased in the steers fed Se (Figures 2 & 3). Plasma Se concentrations increased more rapidly and were generally more labile than whole blood Se. This reaffirms our long-standing position that blood is a better index of long term Se status than plasma or serum in mammals. With the exception of the high $SeO_3^=$ group, blood concentrations appeared to plateau between 60 and 80 days. Dietary selenomethionine resulted in significantly higher blood concentrations than did corresponding dosages of $SeO_3^=$ (Figures 2 & 3). *Post mortem* tissue Se concentrations, with the exception of liver, were also significantly greater with selenomethionine than with $SeO_3^=$ (Figure 4). This differs from the situation in waterfowl where selenomethionine resulted in significantly greater accumulation in liver (Heinz et al., 1988) and likely reflects greater metabolic turnover in mammalian liver.

One steer (#14) developed a conditioned aversion to the selenomethionine diet after 10 days on Se and was replaced in the experiment by a similar animal from a local herd. The aversion exhibited by this steer was so severe that he completely refused hay for several days whether or not it was amended with

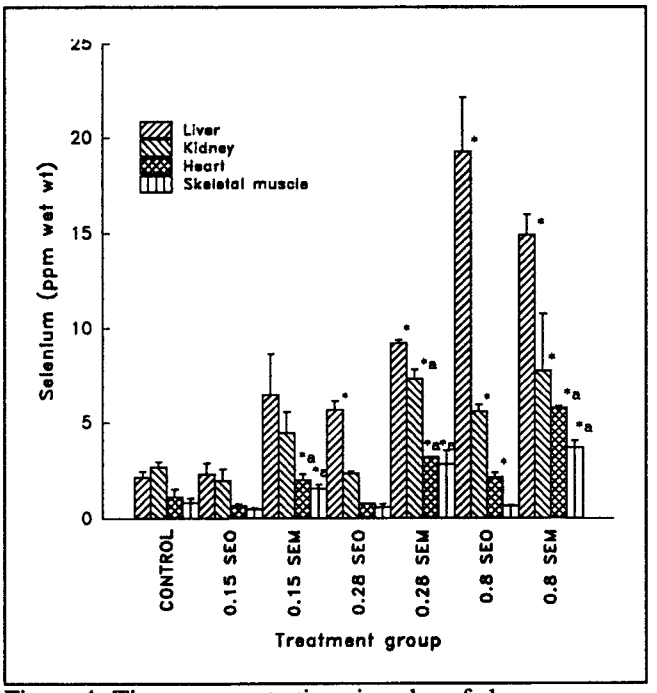

Figure 4: Tissue concentrations in calves fed selenomethionine or selenite at 0.15 mg, 0.28 or 0.8 mg Se/kg BW. "*" indicates significantly greater than controls, "a" greater than the corresponding dose of SeO_3.

Se. This steer was therefore given his daily dose by gavage with a gelatin capsule for the remainder of the experimental period. After being fed by stomach tube for 4 days, it began to eat grass and slowly regained weight, but never completely regained its original condition (Figure 5). We conclude that the difference in blood concentration and effects on body weight was due, in part, to the difference in bioavailability of a single daily gavage dose and continuous feeding.

On day 96, slight swelling and erythema just proximal to the coronary band developed in both front legs. Ten days later a hairline crack appeared parallel to and 0.5 cm distal to the coronary band and the steer became lame. Hoof separation and lameness both increased in severity until, by 120 days, it refused to walk to feed and water and had to be hand fed. At necropsy, new hoof growth was found underlying the separated horny tissue. Histopathologic examination of the juncture between new hoof growth and old revealed hyperplasia and acanthosis of the lamellar epithelium of the hoof.

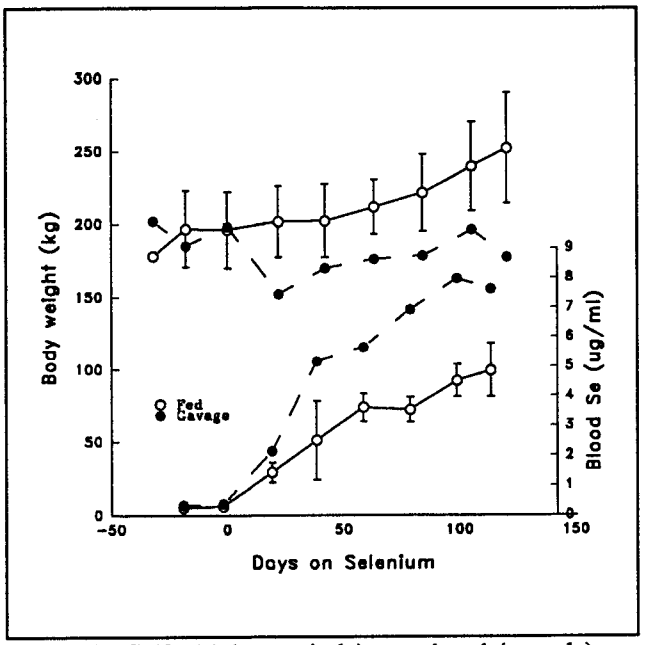

Figure 5: Calf #14 (open circle) was dosed (capsule) with 0.8 mg/kg Se BW as selenomethionine daily, calves in the 0.8 mg/kg Se BW dose group were fed spiked, chopped hay. The initial weight loss was probably due to starvation.

Although none of the calves *fed* Se exhibited any lameness or hair loss, histopathologic lesions similar to those seen in #14 were seen in one high SeO3, one medium selenomethionine and all high dose selenomethionine steers. The dystrophic keratin formation associated with this lesion is probably responsible for the hoof separation seen in alkali disease, and the doses (0.8 mg Se/kg BW as SeO3, 0.28 mg Se/kg BW as selenomethionine) which caused it probably represent a threshold or near-threshold of toxicity. For further description of the lesion and photomicrographs see O'Toole and Raisbeck (1994).

Experimental studies: pronghorn antelope

To validate the results of the steer experiment in a second target species, we undertook a similar study in antelope. Captive-raised male antelope (four yearling fawns, five adults) were housed in concrete-floored pens at the Wyoming Game and Fish Sybille Research Center. Water (less than 50 ppb Se) was provided via automatic waterers and the antelope had access to sheds for shelter from the weather. After a one month acclimation period, the antelope were divided into experimental (two fawns, three adults) and control (two fawns, two adults) groups. The experimental group received a finely chopped 60:40 mixture of 25 ppm Se native grass hay and locally grown alfalfa hay (approx. 0.3 ppm Se). The final Se concentration of the experimental diet was 15 ppm. The control group received a similar diet containing approximately 0.3 ppm Se. The experimental exposure period lasted 168 days.

Although the antelope initially ate the ground grass/alfalfa diet readily, feed refusal of both the control and experimental diets was a recurring problem from day 30 until the end of the study. During the last 60 days of the experiment the diet of fawns was supplemented with 200 gm per day of a corn-oats-barley mixture in order to avoid cachexia. Total Se consumption for the 168 day feeding period was approximately 4100 mg Se/kg BW. Feed consumption and weight gain (Figure 6) were less in antelope on the high Se ration, especially the fawns during the last 60 days of the experiment, which coincided with early Spring. Nonetheless, both groups appeared in good flesh and remained lively and alert throughout the experiment. Specifically there were no signs of lameness, hair loss, vision impairment, or liver, kidney or cardiac abnormalities attributable to the high Se diet.

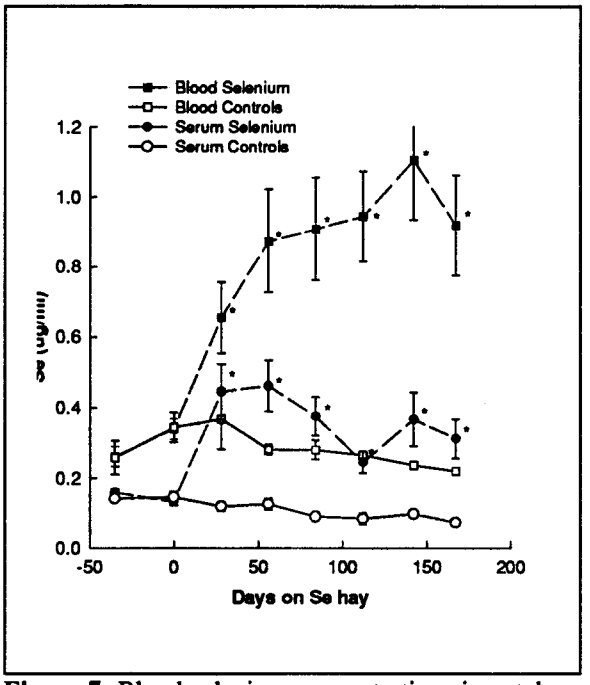

Figure 7: Blood selenium concentrations in antelope receiving 15 ppm hay. Each point represents the mean plus or minus standard error of the treatment group.

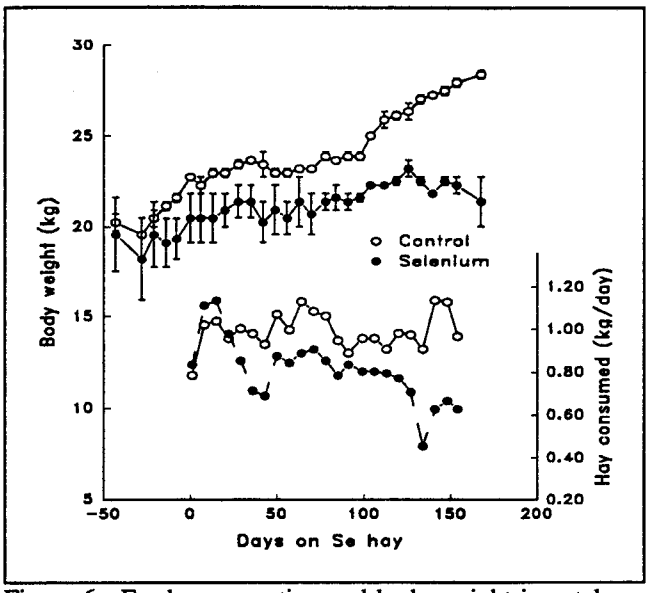

Figure 6: Feed consumption and body weight in antelope fawns on high Se hay. We believe that the lesser feed consumption in the Se hay group accounts for the smaller gain seen.

Clinical pathology and biochemistry testing did not reveal differences between groups, or deviations from historical normals. Blood Se concentrations, while elevated in the experimental group, were intermediate between the bovine $SeO_3^=$ group and the bovine selenomethionine group (Figure 7). Detailed histopathologic examination, similar to that used with the steers, of tissues from the antelope did not reveal lesions attributable to the high Se diet.

Research techniques: caveats

An old graduate school saw indicates that "if the method worked as published, it probably didn't work." The following observations are included for other laboratories interested in Se toxicity and will perhaps spare them some of the aggravation we experienced.

At least part of the confusion about Se toxicity results from radical changes in analytical and biomedical technology during the half century since it was first reported. Studies in the senior author's lab (Dolan et al., 1985), and more recently, by Maas, et al. (1992) indicate that, while various methods for mammalian tissue Se analysis are generally in good agreement, they diverge at either end of the deficiency-toxicity spectrum. Thus, comparison of results between laboratories and, especially, between eras should be undertaken with caution.

In theory, drying samples before inorganic analysis yields more consistent results than running them "wet". However, in our hands, drying a tissue sample such as liver before analysis to obtain "dry weight" Se concentrations resulted in loss of Se. This effect tends to occur with especially high or low Se samples. Often there is insufficient sample to obtain a dry weight/wet weight ratio on a separate specimen, thus we, like most veterinary toxicology labs, routinely use a "live" or wet weight basis for calculating Se concentrations in tissues.

The histochemical technique commonly known as the autometallographic or "Danscher stain" purportedly marks Se in light and electron microscopic sections and has been used in previous reports of experimental Se toxicity (Smyth et al., 1990a, 1990b). Our studies indicate that, while the technique may detect Se in

some tissues, it is insufficiently specific for Se under conditions of chronic exposure for toxicology research (O'Toole et al. 1994a).

Discussion

The results of a 6-year, prospective study of naturally-occurring selenosis in Wyoming indicate that, while chronic selenosis does occur in domestic animals in the state, the incidence is much less than that alluded to by early investigators and the popular press. The clinical presentation of this condition is similar to that originally referred to as "alkali disease", which is characterized by damage to epithelial structures such as skin and hoof. Epidemiologic investigation and our experimental studies suggest that, most if not all, of the other pathologic syndromes attributed to chronic selenosis in domestic animals (eg. anemia, liver damage, cachexia and arthritis) are secondary to the profound lameness seen with selenosis and consequent starvation under range conditions.

To date, we have not verified a single case of the condition referred to as "blind staggers". It is noteworthy that the term "blind staggers" has been historically applied by stockmen to any disease condition of ruminants in which animals exhibit neurologic signs. It is also noteworthy that the signs ascribed by Beath et al., (Trelease and Beath, 1949b) to "blind staggers" are virtually identical to those of polioencephalomalacia, a condition now known to sometimes result from excess dietary sulfur (Raisbeck, 1982). The latter element frequently co-occurs with Se in Wyoming minelands. These results, plus a comprehensive review of archived research materials from the Beath group (O'Toole et al., 1994), lead us to conclude that the condition was mis-diagnosed and probably should have never been attributed to Se.

Our research indicates that higher Se tissue concentrations accumulate when ruminants are fed selenomethionine than when fed $SeO_3^{=}$. The significance of this fact is problematic in several ways. First, what is the diagnostic significance of "x" ppm Se in blood or tissue, when the chemical form ingested is unknown? This may account for some of the discrepancy in the biomedical literature as to what constitutes "confirmed" selenosis. The senior author has seen a case of subacute iatrogenic selenosis in swine in which blood concentrations ranged from 1 - 2 ppm, yet many of our calves tolerated such concentrations for several weeks with only minimal, if any effects. Thus tissue concentrations alone are not sufficient criteria for diagnosis. At present, our criteria are blood concentrations in excess of 1 ppm, *together with lesions characteristic of selenosis*, in the absence of other differential diagnoses. Tissue "levels", do not prove selenosis unless they are greatly (greater than 5 ppm wet weight) elevated. In our opinion, there is serious need for a more specific diagnostic test to confirm selenosis.

Second, what is the toxicologic significance of a given dietary concentration, if the dietary source was selenomethionine? It has been suggested for several years (De Marco and Di Girolamo, 1981; Stadtman, 1991) that dietary selenomethionine accumulates non-specifically in tissue proteins where it poses relatively little hazard. Indeed, Puls (1988) lists quite different diagnostic blood concentrations for "natural" (selenomethionine, presumably from feedstuffs) vs. "selenite" toxicity. On the other hand, results from laboratory rodent and waterfowl studies suggest that chronic dietary selenomethionine is actually more toxic than inorganic Se salts (Fairbrother and Fowles, 1990; Waschulewski and Sunde 1988). The dietary concentrations associated with hoof lesions in our study support the latter hypothesis, but larger scale experiments with more animals are necessary to be completely convincing. Perhaps, selenomethionine's greater bioaccumulation provides a larger pool of Se than e.g. $SeO_3^{=}$ to draw on for the metabolic formation of a proximate toxicant, thus making it *potentially* more toxic after sustained exposure.

The fact that tissue concentrations in antelope fed 15 ppm hay were somewhat lower than predicted by the steer experiment is also problematic, given that Se in grass hay is reported to be predominantly selenomethionine. There are several possible explanations for this observation. It may be that the Se species in our high Se hay was other than selenomethionine. It may be that protein-incorporated selenomethionine is toxicologically different from free selenomethionine. Finally, it is possible that the Se metabolism of

antelope differs slightly from that of cattle. We haven't yet distinguished between these possibilities because of the difficulty in reliably quantitating selenomethionine in hay.

If the difference in the chronic toxicity of selenomethionine and inorganic salts can be accurately quantified for the relevant species, a more precise, predictive reclamation standard for vegetation Se can be based on the proportion of various Se species present. In the meantime, our research suggests that the existing 5 ppm standard is extremely conservative under Wyoming conditions.

Acknowledgements

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