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Investigation of the Toxic Affect of Manganese on Ruminal Microbial Digestion and Growth

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INVESTIGATION OF THE TOXIC AFFECT OF MANGANESE ON RUMINAL MICROBIAL DIGESTION AND GROWTH

by

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Abstract

This research investigated the toxic effect of manganese, in a reduced chemical state, on rumen microbial metabolism and growth. The reduced form of manganese is the soluble state found in subsurface waters and has a high red-ox potential. In the study, we investigated the effects of reduced manganese at varying concentrations, on rumen microbial ability to replicate and metabolize carbohydrates. The hypotheses that we tested were as follows: (1) reduced manganese decreases the rate of pH drop associated with rumen culture fermentation of a corn based diet and (2) reduced manganese decreases the bacterial growth in rumen culture, as measured by bacterial DNA quantification. In this study, rumen culture material was obtained from a rumen canulated cow and dosed with test materials in randomly assigned flasks at 37° C. Cultures were replicated three times on different days. Test materials were dosed to result in the reduced form of manganese at concentrations of 0 ppm, 1.5 ppm, 3 ppm, 6 ppm, 12 ppm, 24 ppm, 48 ppm, and 96 ppm. Samples from each flask were obtained at 0 hr., 3 hr., 6 hr., 9 hr., and 12 hr. for measuring pH and at 0 hr., 3 hr., and 6 hr. for DNA. In these experiments, manganese inhibited the growth of the microbial population at >3 ppm, but the rate of pH drop was not altered among the treatments used in this study.

Investigation of the Toxic Affect of Manganese on Ruminal Microbial Digestion and Growth

Manganese is an essential component of bovine nutrition and has a variety of functions such as utilization of carbohydrates, normal bone formation, normal brain function, growth, reproduction, and a variety of enzyme systems.[1] Some of the enzymatic functions include amino acid metabolism, energy metabolism, enzyme activation, and other metalloenzymes. Manganese deficiency in cattle is associated with retarded/poor bone growth, skeletal abnormalities, enlarged/swollen joints, ataxia of the newborn, reduced birth weight, knuckling of the joints, physical weakness in calves, paralysis, and defects in lipid and carbohydrate metabolism.[2] Manganese deficiency in female bovines also causes disturbed or depressed reproductive efficiency, silent heats, delayed or decreased estrus, poor/reduced conception, abortions, and reproductive failure. Actual expression of deficiency effects varies with the degree of the deficiency and with the timing in which the deficiency occurs.

Manganese is a trace element included at low concentrations for diets of dairy cows.[3] It is usually found in the oxidized form in prepared rations. The systemic absorption of manganese is usually less than one percent, but about 20-ppm manganese in the diet is adequate for growth and satisfactory reproductive performance. The U.S. National Research Council lists the following requirements for manganese 1-10 ppm for growing and finishing cows, 20 ppm for dry pregnant cows, and 40 ppm for dairy cattle.[2]

When manganese is absorbed it is stored primarily in the liver. The liver has been shown to be important in the homeostatic control of certain trace elements, including manganese, and their excretion in bile.[4] Manganese is absorbed in the upper small

intestine and, according to Howes and Dyer, is better absorbed under conditions of low dietary intake.[5] Much of the manganese taken up by the intestinal mucosa of cattle is not transported to the blood but in some way reenters the intestinal contents soon after uptake.[3] Almost all the absorbed manganese is removed from the blood during its first passage through the liver and, in this way, systemic manganese concentrations are maintained at between 0.09 and 0.36 μmol per liter.[6] Bile flow is the main route of excretion for manganese to prevent the accumulation of manganese in the liver.[7] The peak rates of excretion of manganese occur principally around the period of feeding, as indicated by Symonds, Mather, and Hall 1982.[4] Normally, hepatic concentrations of manganese are low and excess absorbed manganese is rapidly excreted into the bile.[7]

The rumen in the cow serves as a fermentation vat where microorganisms, bacteria and protozoa, break down feed into utilizable forms of energy and proteins. Ruminants specifically undergo a process of "rumination" for approximately 8 h/day in order to reduce the particle size of the feed for microbial access.[8] The ingesta, hay, grass, silage, grain mix, water, and saliva, serve as food for the bacteria and protozoa, which break down the feed and produce many utilizable by-products. The microorganisms convert carbohydrates, mainly starch, cellulose, and hemicellulose, to volatile fatty acids (VFAs). Rumen VFAs are absorbed from the rumen and are the major source of energy for the cow. The bacteria break down protein to keto acids and ammonia. The microbes then use the ammonia and keto acids to rebuild protein for themselves as they grow. As the microbes die and pass out of the rumen, the animal digests these microbial proteins and use then for their own growth and development.[9] Manganese in an oxidized form reacts with bacteria in a harmful way at concentrations of over 1000 ppm. At these

concentrations, it causes depression of volatile fatty acid production, especially propionate, and can result in marked changes in the rumen flora.[10]

Manganese toxicosis is characterized by to reduced appetite, weight gain, fiber digestibility, bile flow, hemoglobin values, and growth rate.[7] Additionally, excess manganese can interfere with iron metabolism, resulting in anemia and abdominal discomfort/pain.[11] Manganese also affects other elemental concentrations due to the interaction competition between other elements. High manganese causes calcium retention in the fetus, increased iodine excretion, reduced iron absorption, and reduced excretion of copper with subsequent increase in liver copper. Male calves (130 days old) fed diets containing 1000 ppm manganese for 18 days showed no signs of toxicosis, while depressed feed intake and lowered body weight were observed in calves fed a low-manganese ration supplemented with manganese sulfate at levels of 2460 and 4920 ppm manganese. When supplemented at a level of 820 ppm, no effect on growth or appetite was apparent. The adverse effects of excess manganese on growth were shown to be mainly a reflection of depressed appetite.[2]

A study of excess dietary manganese and feed lot performance of beef cattle showed significant insight to how excess manganese affects rumen microbes.[12] Conventional digestibility investigations and clinical changes in tissues and peripheral blood were employed to study effects of excess manganese upon feed lot performance of 48 yearling Hereford calves. Animals were maintained in four lots for 160 days on a corn, CSM, molasses and Bermuda grass hay ration, supplemented with 0, 250, 500, or 1000 ppm manganese ($MnSO_4$). Results showed that the ability of rumen microorganisms to digest cellulose *in vitro* was markedly reduced in animals on high

manganese diets. The data indicates the effects of excess manganese to be greater on function than structure of the rumen.

Manganese has recently been found of high concentration in the drinking water of some dairy herds. These herds have shown signs of significant weight loss, low water intake, poor milk production, and anorexia. The manganese in well waters is in the reduced form, because oxidized forms of manganese are very poorly water-soluble. A recent study with reduced iron showed decreased rumen microbial digestion and growth. Manganese, in the reduced form, is even more reactive than iron and may do more harm at lower concentrations. It may be possible that manganese just tastes bad and affects the cow's appetite, because trace concentration of 0.05 ppm result in poor palatability of drinking water in people.[13]

Water is a very important nutritional requirement that is often overlooked in the management of livestock. In ruminants, the daily dietary dry matter (DM) intake is about 1.5-5% of their body weight. In comparison, their daily water intake is about 10-15%.[14] Although factors such as environmental temperature and forage type affect the total daily water intake, ruminants generally consume 3 to 5 times more water than DM daily. Thus, materials in water would be expected to reach toxic doses at lower concentrations than in the DM portion of the diet.[13]

With any element there is possibility for overdose and the development of toxic effects. A nutrient may be essential to an animal in small amounts, but when taken in excess it may result in toxic effects, mild or sever. If water contains toxins in sufficient quantity, the cow's health may be adversely affected and death is a possible outcome. In this study we want to determine if reduced manganese as is found in water can affect the

rumen microbes so they cannot reproduce and/or break down food. Also, we will determine at what concentration of reduced manganese the microbes are affected. The hypotheses that will be tested are: (1) reduced manganese will decrease the rate of pH drop associated with rumen culture fermentation of a corn based diet source and (2) reduced manganese will decrease the bacterial growth in rumen culture, as measured by bacterial DNA quantification. We will test these hypotheses by taking pH readings of eight different concentrations of reduced manganese (0 ppm, 1.5 ppm, 3 ppm, 6 ppm, 12 ppm, 24 ppm, 48 ppm, and 96 ppm) at five separate time points (0 hr., 3 hr., 6 hr., 9 hr., and 12 hr.) and by calculate bacteria growth by DNA quantification via the first three time points. The objective of the study is to find a correlation between the reduced manganese and the symptoms of the cattle to provide causation for the clinical syndromes observed. By using rumen culture methods, we will eliminate the possibility of taste and poor water intake as other possible causes of effects.

Materials and Methods

Reagents, Fluids, Buffers:

Chemicals. The following chemicals were purchased from Sigma Chemical Co.: cysteine*HCL, Antifoam 204, and DNA standard s. The chemicals obtained from Mallinckrodt were: H_2SO_4, diphenylamine (DPA), $NaHCO_3$, $NaHPO_4$, $MgSO_4$, NaCl, and $CaCl_2$. The chemicals $CoCl_2$, NaOH, and acetaldehyde were acquired from Acros Organics. KCL and perchloric acid were purchased from Baker. The chemicals bought from Fisher Scientific were: ethylene diamine tetraacetic acid (EDTA), $NaS*9H_2O$, and $MnCl_2$. FeCl3 was purchased from Aldrich and glacial acetic acid from EM Science.

Incubation Reagents and Buffers. McDougall buffer consists of 39.4 g/L $NaHCO_3$, 14.8 g/L Na_2HPO_4, 2.28 g/L KCL, 1.88 g/L NaCl, 0.48 g/L $MgSO_4*7H_2O$, 0.56 g/L $CaCl_2*H_2O$, and 0.56 ml micromineral solution.[15] Add ingredients singly. Once materials are dissolved, bubble CO_2 through the buffer 30 min. to 1 hour. Then, take pH reading again and adjust to below 6.8 with HCL. Afterwards, bring the solution to a final volume. Keep the solution at 37 ˚C if it is going to be used within a few days, otherwise, store it at room temperature for up to 3 months.

The reducing solution contains 4.2 g cysteine*HCl, 12.9 g Na_2S, and 100 ml dH_2O. The micromineral solution consists of 3.54 mM $MnCl_2*4H_2O$, 0.294 mM $CoCl_2*6H2O$, and 2.07 mM $FeCl_3$. Dissolve and bring to final volume with ddH2O. This solution is stored in the refrigerator. It is very stable, and has a very long shelf life, many years.

DNA Reagents. The DNA standard s standard is prepared by dissolving 10.0 mg of DNA/ml of 5 mM NaOH and stored in the refrigerator. The diphenylamine (DPA)

reagent consists of 30 g of DPA and 30 ml 36 N H_2SO_4 in 2000 ml glacial acetic acid.[16] This is prepared in advanced and stored at room temperature until needed. The EDTA is forced into solution by applying heat and stirring while titrating with 1 N NaOH until the pH reaches approximately 8.4.

Culture preparation:

The *in vitro* experiment involved several preparation steps. This protocol was developed by Broderick.[17] All reagents are prepared in advance. Add 3.0 g of corn to each flask and put in the incubator set at 39 ˚C. Labelled 12 x 75 mm tubes for each time point and flask number had 200 µl of 2.75 N Perchloric acid added to each tube. The large Erlenmeyer flask, large graduated cylinders, large funnel, prepared buffer, and tip flask were placed into 39-˚C oven.

Morning of the Experiment:

The collection thermos was filled with tap water that was as hot as possible and the lid placed securely. Antifoam was sonicated for 10 min. and 2.0 ml added to each incubation flask, then the flask was placed back into the incubator. The perchloric acid containing 12 x 75 mm tubes were placed on a bed of crushed ice.

Rumen Fluid Collection:

Rumen material was collected from a cannulated, lactating dairy cow prior to being fed on the morning of each incubation replicate. The rumen contents was collected manually and placed into a water thermos that had been filled with hot water until the

time of collection. A plastic 150 ml beaker was used to collect the rumen samples and a total volume of 750-800 ml of rumen fluid was placed into the thermos. The rumen contents were topped with a layer of fibrous feedstuffs and the lid screwed on tightly to prevent the introduction of oxygen while transporting the material to the lab.

Preparing Inoculum for *In Vitro* Experiment:

Collected rumen materials were filtered through four layers of cheesecloth while under continuous CO_2 perfusion. A volume of the McDougall buffer equal to the collected rumen filtrate was poured over the rumen fiber in one third increments, squeezing the liquid through the cheesecloth, and combining it with the original rumen fluid. Resultant mixture of 1:1 (v/v) rumen fluid: McDougall buffer is the rumen inoculation material.

Reducing solution and antifoam 204 solution (diluted 1:50 in dH_2O) were both added to the rumen inoculation material at 1% of the total volume. Rumen inoculation material was maintained at 39 °C and perfused continuously with CO_2. Using a 100ml-graduated cylinder from the oven, 90 ml of rumen inoculation material was dosed into each spinner flask. This was done while keeping the CO_2 flowing over the cylinder, and into each flask. Next, the experimental test concentrations of manganese were randomly added to the flasks. Treatment time and pH of the each flask was recorded immediately after adding the rumen inoculation material, which represented the time zero measurements. Then, two-2ml aliquots were placed into pre-labeled 12 x 75 mm tubes. Flasks were returned to the incubator and verified that they were spinning. This procedure was repeated until all the flasks were dosed. The 12 x 75 mm tube of

perchloric acid/rumen fluid was placed into a 10°C freezer until analyzed.

The pH measurements and collection of samples into perchloric acid was then repeated at 3 hr, 6 hr, 9 hr, and 12 hr. CO_2 was perfused into each flask as sampling was performed, until reseated and placed back into the incubator.

pH:

The pH of each flask was measured directly in each flask prior to the sample collection for DNA analyses at all time points. An Orion model 370-pH meter was used for pH measurement.

DNA Analysis:

The following protocol was developed by Burton.[16] The vials of 2 ml aliquots were thawed and 1 ml of 70:1 EDTA was added to each sample. Samples were vortexed and refrigerated for one hour, then centrifuged at 12,000-x g at 4 °C for 20 minutes. The supernatant was then discarded. Pellets were re-suspended in 1.0 ml of 0.5 N perchloric acid (PCA) using a glass rod which was rinsed with an additional 1.0 ml of 0.5 N perchloric acid, and the suspension was vortexed. Then, the samples and standards were hydrolyze in a 70 °C water bath for 30 minutes, with all the tubes being vortexed every 7 minutes throughout the hydrolysis.

Tubes were cooled and centrifuge at 12,000 x g (4 °C) for 20 minutes. Then, the supernatants were transfered to graduated 12 x 75 mm tubes. The pellets were resuspended in1 ml of 0.5 N perchloric acid with a glass rod, recentrifuged, combined with the previous supernatant, and vortexed. Each tube was brought to exactly 4.0 ml

with 0.5 N PCA. DNA was measured by the method of Burton.[16] 1.0 ml from each sample tube was pipetted into the appropriately labeled 13 x 100 or 16 x 100 glass tube. Then 2.0 ml of the final DPA reagent was added, the mixture vortexed, and the tubes covered with foil. Prepared analyzed samples were left on the bench overnight for the reaction to proceed. Tubes were read at 600 nm. If tubes were cloudy, they were centrifuged again to remove interfering protein.

Statistical Analysis:

Variables analyzed statistically were rate of pH change from 0 to 12 hours and the change in deoxyribose nucleic acid content (DNA concentration) of the samples from 0 to 6 hours. All variables are based on per incubation flask activities as previously described. All statistics are calculated using mixed model ANOVA methods. Significance was set at $p < 0.05$.

Results and Discussion

The rate of pH drop in the rumen cultures differed, dependent on the time points of testing (Figure 1). The earliest measurements, 0 to 3 hr, had smaller slopes than those of 3 to 6 hrs or 6 to 9 hrs. But, there were no significant differences among the treatments for any of the time periods. The slope was shallower for the 9 to 12 hour period, as would be expected, due to build-up of metabolic waste, microbial deaths, and exhaustion of metabolic inputs.

In ruminants, the primary source of protein comes from the degradation of rumen-produced microbial mass. Therefore, decreases in the growth of the rumen microflora not only would limit the amount of fermentation that occurs, but also decreases the amount of microbial protein available to the animal. Also, in this type of incubation, pH correlates highly with the metabolism of the microbes. Particularly, pH has a negative correlation with microbial metabolic acid production. As pH of the incubation medium declines, organic acids are increasing. Therefore, the rate of decline in pH over time is a good indicator of total metabolism of the microflora, assuming that the initial pH and buffering capacity of the incubation flasks are all equal. This is of importance in ruminants because these metabolic acids, primarily VFA, produced by the rumen microbial population are one of the primary energy sources for the animal.[13]

In these experiments, the pH was not a good indicator of manganese toxicity, as there was no measured change in microbial digestion. The rate of pH drop did not differ among the treatments used in this study (Figure 1), indicating that the digestion of the bacteria is not affected by the higher concentration of manganese. These results differ from those expected, based on previous work with the reduced form of iron. The

reduced form of iron significantly inhibited the drop in pH associated with carbohydrate metabolism in rumen cultures.[13] Thus, if the reduced form of manganese alters rumen function, the mechanisms of effect are at least somewhat different than those of iron.

Manganese in the reduced form has greater red-ox potential than the reduced form of iron.[13] The high red-ox potential of reduced manganese would indicate the high potential for damage to cell membranes and thus the loss of microbial metabolic capability. It is possible that the reduced manganese was so reactive that it reacted with or bound other macromolecules in the culture material before it could react with the microbial membranes in a significant manner.

Statistical elevation of the DNA quantification could not be performed on this experiment because one replicate of DNA samples was lost to analysis. This left only two DNA samples to be processed for each treatment time point. Manganese was found, though, to have a general trend of decreasing the rate of microbial growth from 0 to 3 hours when administered at concentrations of 12 ppm, 24 ppm, and 96 ppm (Figure 2). In comparison, decreased microbial growth appeared to occur at concentrations of 3 ppm, 6 ppm, 12 ppm, 24 ppm, 48 ppm, and 96 ppm for the 0 hour to 6 hour period (Figure 3). The comparison between the 0 to 3 hr and 0 to 6 hr incubation periods would indicate that the Mn required time in culture in order to produce the most dramatic observable effects. In the live animal with continual intakes, incubation, and turnover, this could result in a significant effect on microbial populations. From 0 ppm to 3 ppm int the 0-3 hr time period, an increase in the DNA was observed which would be an indicator that the bacteria may be incorporating the Mn into the system in a beneficial way, but this incorporation may be ultimately detrimental based on the 0 to 6 hr time period data. At

48 ppm there appeared to be an anomaly because the concentration means increased for both time periods (Figures 2 and 3), but the general trend was of a decrease in growth rate.

The problem with high concentrations of manganese in rumen cultures likely involves an inhibitory effect on bacteria replication. This could involve killing the bacteria or interference with new DNA synthesis during replication. In a study done by Beckman, it was shown that at higher concentrations of manganese (between 500 - microM and 1.5 mM) Mn-deoxynucleoside triphosphate interactions contribute to mutagenesis. The concentration dependence of mutagenesis is determined by the strength of binding manganese has to the particular DNA template used. [18] With a loss of microbial replication, a ruminant would lose valuable nutrients because of lesser microbial proteins, as well as potentially smaller numbers of microbes to digest carbohydrates as microbial populations decline over time. Thus, without the microbial nutrients, a cow could lose weight or have poor feed efficiency, as has been shown in previous dosing studies.[2] This potential is supported by the trend towards decreased microbial growth rates in this study, for 3 ppm to 96 ppm. Further studies to more precisely outline the degree of adverse microbial effects, as well as investigate the toxic mechanisms for manganese in water, are indicated by the results of this study.

Figure 1: Presentation of the pH measurements across time for rumen cultures dosed with varying concentrations of manganese

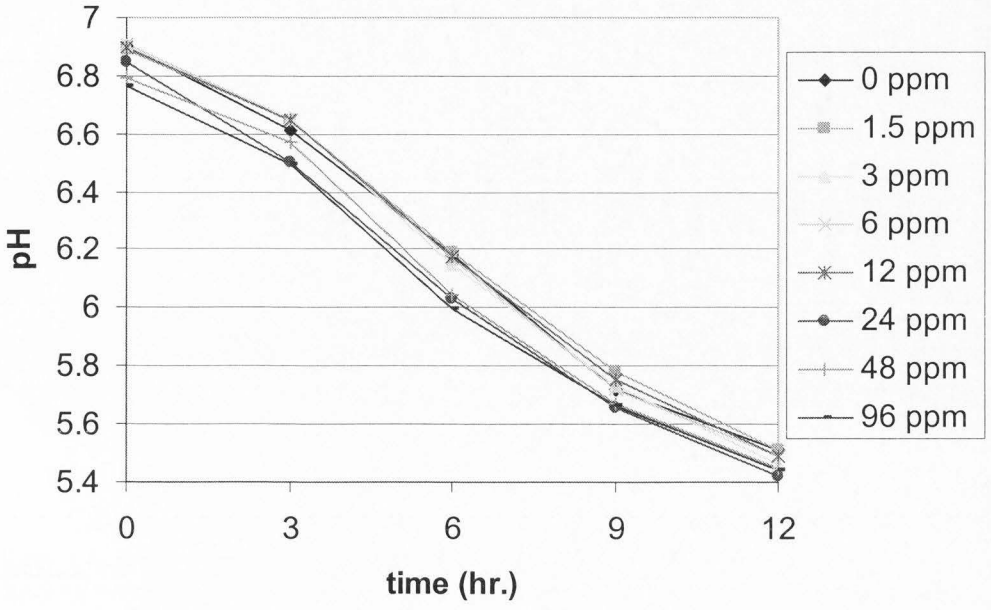


Table 1. Mean microbial growth rates (mg DNA/ hr) for rumen cultures supplemented with varying concentrations of MnCl2. Changes in DNA concentrations for cultures with 0 ppm, 1.5 ppm, 3 ppm, 6 ppm, 12 ppm, 24 ppm, 48 ppm, and 96 ppm MnCl2 were measured from 0-3 hours and 0-6 hours

TRT (ppm)	0-3 hr mean (±SE)	0-6 hr. mean (±SE)
0	0.01128	0.00597
1.5	0.01259	0.00698
3	0.01575	0.00622
6	0.01384	0.00552
12	0.01034	0.00503
24	0.00927	0.00352
48	0.01216	0.00441
96	0.00795	0.00304

Figure 2: Rate of increase of microbial DNA from rumen cultures incubated for 3 hours with varying concentrations of manganese in a reduced chemical state.

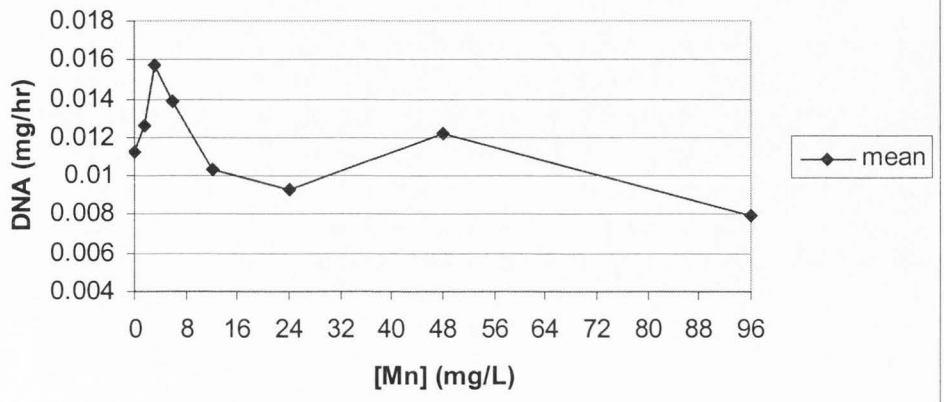
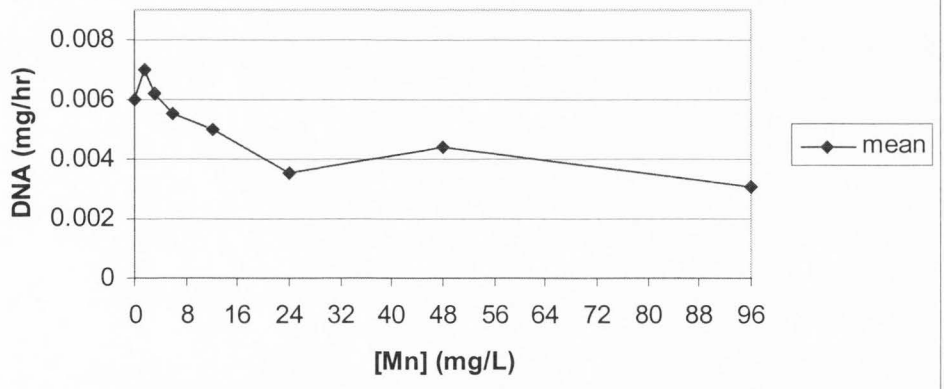


Figure 3: Rate of increase of microbial DNA from cultures incubated for 6 hours with varying concentrations of manganese in reduced chemical state.



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