AN ABSTRACT OF THE THESIS OF

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A series of factorial experiments (4 x 4 and 4 x 4 x 4) was designed to investigate the effects of selected combinations of some major and trace elements upon in vitro rumen cellulose digestion. Inocula, varying in ratios of clarified rumen fluid (strained through No. 50 cheesecloth and centrifuged at 365 x g for 2 minutes) to basal mineral medium to phosphate buffer, were incubated at 39 degrees C with purified cellulose and the corresponding treatments of test elements. After a 24-hr incubation period, cellulose digestion was calculated. In addition, the mineral composition of rumen fluid and rumen bacteria was studied in relation to the mineral composition of the diet of a fistulated steer.

Mineral analysis for 6 major and 15 trace elements clearly indicated that, when compared to the diet, concentrations of these elements in the clarified rumen fluid were several times lower, whereas concentrations in rumen bacteria were several times higher. In general, rumen bacteria appeared to concentrate trace elements to a greater extent than major elements with approximately the following overall distribution: elements concentrated in excess of 20 times included Co; 10 to 20 times--Se; 5 to 10 times--Na, P, Al, Fe, Mo; 1 to 5 times--Ca, K, Mg, S, B, Ba, Cd, Cu, F, Mn, Ni, Sr, and Zn. Chromium was the only element found to be in lesser concentrations in rumen bacteria than in the diet.

Significant interactions ($P < .05$) affecting *in vitro* rumen cellulose digestion were found amongst the following combinations of elements: Mn-Zn, Ca-Zn, Mg-Ca, Mn-Fe, S-Mg-Ni, P-Mg-Ni, Mg-Co, P-Mg-Co, Mg-Co-Ni, and Cu-Mo-S. Additions of non-toxic levels of Mn partially but significantly reversed the inhibition in cellulose digestion caused by additions of excessive Zn. This protective effect remained significant when ratios of clarified rumen fluid to basal mineral medium to phosphate buffer were altered. Similarly, additions of Ca to the incubation medium significantly ($P < .05$) protected against the toxicity of excessive Zn. The significant ($P < .05$) depression caused by excessive Ca, on the other hand, was partially but significantly reversed by additions of Mg. Added Fe (only at levels

of 10 μg/ml) had some protection against the depression in cellulose digestion caused by excessive Mn.

Additions of 0.1 percent NaCl were effective in counteracting the depression in cellulose digestion caused by excesses of KCl, however, additions of larger quantities of NaCl in the presence of 1.0 percent KCl resulted in total suppression of cellulose digestion.

Significant interactions ($P < .05$) were found between S, Mg and Ni. Additions of non-toxic levels of Mg partially reversed the severe depression in digestion caused by excessive Ni with additions of S or P significantly enhancing this protection. Non-toxic levels of Mg were also successful in alleviating the depressing effects of excessive Co. Additions of S or P, however, were ineffective in augmenting the protection offered by Mg. When Mg, Co and Ni were tested simultaneously, Mg offered the already established protection against Ni and Co individually as well as against combinations of the two elements. However, as the concentrations of Ni and Co increased, the depression in cellulose digestion also increased, but the protection offered by Mg decreased.

Copper was found to be highly toxic to rumen microorganisms (additions of 0.5 μg/ml practically stopped all microbial activity). Neither Mo nor S or both were effective in alleviating the depression caused by excessive Cu. In lieu, the simultaneous presence of the three elements, in concentrations in which individually were non-

deleterious to cellulose digestion, proved toxic to cellulose digestion. Nonetheless, additions of Mo significantly ($P < .05$) increased digestion in the absence of added Cu and S.

A S requirement by rumen microorganisms for optimum cellulose digestion was established in three separate experiments. A level of 8.4 μg/ml S--present in the inoculum--did not support adequate cellulose digestion, whereas, additions of 10 μg/ml S (18.4 μg/ml total S) to the inoculum resulted in maximum digestion.

Effect of Some Major and Trace Element Interactions Upon In Vitro Rumen Cellulose Digestion

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INTRODUCTION

A recent FAO publication (1970) reveals some interesting facts on the importance of ruminants to the welfare of man. Production of meat from domesticated ruminants, for example, accounts for roughly half of the world's production, while production of milk from ruminants accounts for practically the entire world milk production. Furthermore, in the past 18 years meat production from ruminants has risen by about 80 percent and milk production by 50 percent. From these statistics one may speculate and infer that the trend in increased productivity is to continue for some decades or, at least, until a suitable protein source replaces ruminant meat and milk.

Sources of protein for humans, other than animal protein, have not yet been successfully processed and manufactured in any substantial volume, their principal drawbacks being palatability and texture. Soya bean "meats" and "milks" as well as some high protein cereal varieties, however, show some promise, probably not entirely as meat replacers but as principal sources of protein, particularly in the developing countries.

Because of the microflora and microfauna that inhabit the rumen, the ruminant animal can utilize cellulose, non-protein nitrogen, waste products of food processing (e. g. sugar-beet tops and pulp, straw, molasses, distillers grains, organic acids, etc.) and, probably most important, they can graze on lands not suitable for farming. The conversion of these poor quality feedstuffs into highly desirable foods such as meat and milk challenges the nutritionist to develop a better knowledge and understanding of the relationships between the host and the commensals and in particular to a more complete knowledge of the nutrient requirements of both.

Much of the present knowledge of the nutrition of rumen microorganisms has been attained through the use of in vitro rumen fermentations. Although these in vitro techniques do not fully emulate the intricate rumen environment, they have been successfully used in ascertaining the energy and nitrogen needs of the rumen microbiota and to a lesser extent their needs for vitamins, minerals and other growth promoting substances. Because of the relatively low cost, the rapidity with which results can be obtained and because of the large numbers of treatments that can be tested in a single run, in vitro techniques are well suited for the study of nutrient interactions affecting rumen microbial activity.

Amongst nutrient interactions, some of the most interesting and sometimes most difficult to understand are those occurring be-

tween mineral elements. If the amount of one mineral element required for maximum performance depends on the concentration of another mineral or nutrient or combinations of nutrients or combinations of minerals, the exorbitant number of possible interactions would make it a tremendous task to test all combinations. Therefore, when investigating mineral interactions, use is first made of the atomic configuration of the elements to theoretically predict the existence of interactions. This is followed by biological experimentation. Then, if two elements possess similar ionic radii, similar structure of the valence shell of electrons, favor similar coordination numbers and tend to form similar complexes (square, tetrahedron, etc.), it is likely that the elements (ions) will interact antagonistically. Cuprous and zinc ions, for example, have identical structure of the valence shell of electrons; possess a favored coordination number of four; and both ions tend to form tetrahedral complexes. Upon biological experimentation (with chicks), these ions indeed interact with the net result of Zn enhancing the severity of Cu deficiency or Cu protecting against excessive Zn.

Few mineral interactions affecting rumen microbial activity have been reported. These have dealt primarily with minerals that are known to form complexes or elements that block certain reactions. This study, therefore, attempts to establish selected mineral inter-

actions affecting in vitro rumen cellulose digestion based primarily on the prediction by the atomic configuration of the elements.

REVIEW OF LITERATURE

Since the mineral nutrition of rumen microorganisms has been extensively reviewed recently by Hungate (1966), Church (1969) and the author (Martinez, 1969), no review will be made of each individual element. Rather, this review will cover, more specifically, the mineral composition of rumen fluid and rumen microorganisms and the mineral interactions affecting the activities of the rumen microbiota.

Mineral Composition of Rumen Fluid and Rumen Microbes

Major Elements

The importance of some of the major elements in the maintenance of adequate osmotic pressure, acid-base balance and redox potential has been delineated by Brouwer (1961) and by Keynes and Harrison (1970). In addition to the contribution in maintaining an adequate rumen environment for microbial growth, the major elements perform other vital functions within and without the microbial cells (see Church, 1969; Hungate, 1966; Martinez, 1969). Studies on the concentrations of major elements in rumen fluid are diverse and somewhat numerous, therefore, only those that deal in their entirety with major elements will be discussed.

Poutiainen (1970) investigated the concentrations of major elements in rumen fluid and their changes between feedings. His data indicated the following patterns: dry matter intake or proportion of long hay in the ration had no effect upon the concentrations of Na, K, Cl, Mg or Ca, but decreasing the dry matter intake increased P concentrations; time of sampling after feeding significantly affected the concentrations of Na, K, Ca, Mg and Cl but not those of P; the sampling point in the rumen affected the concentrations of all the elements tested. Levels of intake as well as time of sampling after feeding were also shown to influence the concentrations of Na, K, Ca, Mg and P (Fenner, Dickinson and Barnes, 1969; Fenner and Damon, 1969). The passage of major elements from the reticulorumen has been reported by Poutiainen (1968, 1971) and Lampila (1965), while the distribution of soluble Ca and Mg and of inorganic P in the various subdivisions of the stomach of sheep on different diets was examined by Garton (1951). Some relationships between the concentrations of Na, K, Cl and P in mixed saliva and rumen fluid have been explored by Bailey (1961).

Evans and Davis (1966a) found that levels of inorganic P and S were much lower in rumen fluid than in the diet, however, such fluid levels rose with dietary increments of the elements. Similarly, Upton and L'Estrange (1971) and Spais, Lazaridis and Agiannidis (1968) showed that total S levels in rumen fluid increased with dietary S

increases. Increases in dietary K also resulted in increased levels of this element in rumen fluid (Devlin, Roberts and St. Omer, 1969).

It appears, from the preceding discussion, that although some general patterns are observed in the levels of major minerals (increase of the elements with dietary increments, differing concentrations in distinct locations of the rumen, etc.), quantitative predictions and/or comparisons between several investigations leave much to be desired.

Information on the major mineral composition of rumen microorganisms is not readily available in the literature.

Trace Elements

As with the major minerals, concentrations of some trace elements (Co, Cu, Mn and Zn) in rumen fluid tended to increase with dietary increments of the particular element (Zerebcov, Vrakin and Sevelev, 1971b). Cobalt increased in rumen contents from 1.32 to 2.86 to 11.80 μg/100 g when Co-deficient hay, adequate hay, and Co-deficient hay supplemented daily with 1 mg Co, respectively, were fed to sheep (Tosic and Mitchell, 1948). In addition, it appears that some elements influence the concentrations of others in the rumen fluid. Mills (1960), for example, suggested that Mo and S depressed the levels of soluble Cu in ovine rumen and abomasal fluids, however, such interaction was not corroborated by Evans and Davis (1966a).

Nonetheless, Zerebcov et al. (1971b) found that supplementation with Cu, Mn or Zn resulted in an increase in the concentration of Co in the rumens of bullocks. Some changes in the concentrations and solubilities of Mn, Cu and Zn in the rumen, abomasum and small intestine of the ovine have been examined by Bremner (1970).

Trace element levels in clarified rumen fluid have been reported by Sala (1957) to be on the order of (ppm): Co, 0.06; Cu, 0.36; Fe, 2.25; Mn, 1.90; Mo, 0.076; and Zn, 1.17. Zerebcov et al. (1971a) found that rumen fluid obtained 3 hours after feeding contained, in mg/liter: Co, 0.03; Cu, 0.11; and Mn, 0.1; levels of Fe in the rumen fluid were approximately 1 ppm in analyses carried out by McNaught and Owen (1949).

Cobalt has received much attention in the past 30 years because of its integral part in vitamin B_{12}, which is synthesized in the rumen by rumen microorganisms. To this effect, Marston, Allen and Smith (1961) concluded that when the concentration of Co in the rumen contents of a sheep fell below 40 mμg Co/g (20 μμg/ml of the bacteria-free supernatant), the synthesis of vitamin B_{12} was significantly reduced.

Trace elements appear to be concentrated, to various extents, by rumen microorganisms when compared to levels in the diet. Tosic and Mitchell (1948) reported that ovine rumen microorganisms concentrated Co from their external environment to the extent that about 80 percent of the Co in the rumen contents was found in the microbes.

Their data also showed that when hay containing 0. 07 ppm Co (dry matter basis) was fed to sheep, the rumen microbial fractions attained a level of 3. 62 ppm. Likewise, Se has been found to be concentrated two- to ten-fold by rumen bacteria in one experiment (Whanger et al., 1970), whereas, in another experiment, Whanger (personal communication) found that Se was concentrated, on the average, 18 times. Iron appears to be concentrated by rumen microbes upon in vitro incubation (McNaught, Owen and Smith, 1950). Elements that have been reported to be concentrated some ten times by rumen microbes included Cu, Ni, Mo and Zn, while V, Ti, Mn and Fe were concentrated to a lesser extent (Mitchell and Tosic, 1949).

Regarding the ash content of dried rumen bacteria, Ellis et al. (1958) found it to be 9. 41 percent. This ash contained, in approximate ppm: Cu, 100; Co, trace; Fe, 300; Mo, 10; and Zn, 200. However the Mo content of the dried, fat-free cells was about 1 ppm.

Mineral Interactions Affecting Rumen Microbial Activities

Few mineral interactions affecting some phase of rumen microbial activity have appeared in the literature. Reports on these interactions have originated in various laboratories under diverse fermentation conditions, therefore, each report will be treated separately.

The interaction between S, Cu and Mo in the ruminant animal has received much attention in the past two decades. The interaction of these elements in the rumen ecosystem, however, has not been fully explored and reported data are in disagreement. Appropriate combinations of levels of Cu and Mo appeared to have an additive effect in increasing cellulose digestion (Ellis et al., 1958). Added S reduced the depression in cellulose digestion caused by excessive Cu, while in the presence of high levels of P, the addition of Cu or Mo lessened the depressing effect of the other (Evans and Davis, 1966b). In contrast, Zembayashi, Kawashima and Uesaka (1968) found that additions of Cu did not alleviate the depression in rumen protozoal VFA production caused by excessive Mo; rather, Cu promoted Mo inhibition. Their data also indicated that added S enhanced Mo toxicity, while P alleviated the inhibition of Mo on VFA production. Furthermore, additions of Cu were ineffective in ameliorating the inhibition caused by excesses of either Mo or S. Kawashima, Uesaka and Toyama (1968) have reported on a Mo-SO_4-P interaction affecting H_2S production by rumen bacteria. Apparently, excess Mo inhibited the H_2S production in the presence of SO_4, action which was enhanced by the presence of P in the medium.

High levels of Mo added to a cattle ration significantly depressed the synthesis of Vitamin B_{12} (Davis, Jack and McCall, 1956). How-

ever, additions of Co to this high Mo ration were beneficial, apparently because of an increased vitamin B_{12} synthesis by the rumen microorganisms.

An interesting interaction between F, Mg and Mn ions affecting in vitro rumen cellulose digestion has been described by Chamberlain and Burroughs (1962). Their results indicate that excessive F as well as omission of Mg and Mn caused a depression in cellulose digestion, but additions of Mg appeared to increase the resistance to F toxicity.

Amongst the major elements, a Na-K interaction affecting in vitro cellulose digestion was uncovered by Hubbert, Cheng and Burroughs (1958a). Additions of Na had either no influence or depressed cellulose digestion, but in the presence of appropriate concentrations of K, added Na increased cellulose digestion. In the same study, it was found that Rb but not Li or Cs could replace about 50 percent of the K requirement for in vitro rumen cellulose digestion.

Uesaka et al. (1968) have studied in detail the interactions of Ca-P, Ca-Mg, P-Mg and Ca-P-Mg as they affect cellulose digestion by mixed rumen bacteria. Their findings indicated that, in the presence of Ca, the depression caused by excessive P was significantly reduced, whereas, added Mg clearly alleviated the depressive effect of excessive Ca or P added individually or in combination.

Interactions between major and/or trace elements and other nutrients have also appeared in the literature. A recent report by Kim, Kawashima and Uesaka (1969b) noted that the toxicity of a variety of trace elements upon in vitro rumen cellulose digestion was enhanced by addition of certain unsaturated fatty acids. It appears that some amino acids and proteins either exert a protective effect against the toxicities of some trace elements (Co, Cu, Se and Zn), have no effect (Fe and Mn) or augment (F and Mo) such toxicities (Kawashima, Kim and Uesaka, 1969; Kim et al., 1969a).

From the foregoing discussion it is apparent that the rumen ecosystem is quite complicated and that changes in the intricate balance of nutrients are going to affect the activities of the rumen microorganisms. Evidently, when evaluating research of this nature, it is imperative that all possibilities be exhausted before final judgment is made.

EXPERIMENTAL PROCEDURE

Mineral Analyses

Preparation of Samples

Grass Hay. Samples of poor quality grass hay, which constituted the sole diet of the fistulated steer (rumen fluid donor), were taken from several bales, ground through a No. 60 wire mesh in a Wiley mill, dried in an oven at 60 degrees C. for 48 hours and stored in hermetically sealed containers until time of elemental analysis.

Rumen Fluid. Rumen fluid was collected from a permanently fistulated Hereford steer consuming the above mentioned grass hay. The rumen fluid was strained through 4 layers of No. 50 cheesecloth and placed in a 39 degrees C. water bath for approximately 30 minutes or until it separated well into 3 layers. From the center or middle fraction 200 ml were removed by means of suction and immediately centrifuged at 1, 500 rpm (365 x g) for 2 minutes. The supernatant (clarified rumen fluid) was then frozen while the sediment, which consisted of small grass particles and protozoa was discarded. Five collections were made in a period of 2 weeks with all samples being pooled and then analyzed. For analyses of F, S, and Se the clarified rumen fluid was first freeze-dried.

Rumen Bacteria. In quantities of 8 to 12 liters, rumen fluid was collected and processed as above excluding the freezing of the clarified rumen fluid. The fluid was centrifuged in a continuous-flow Sharples super centrifuge with a flow rate of approximately 3.5 liters/hr and a speed of about 23,000 rpm. The semi-solid sediment (bacterial fraction) was then transferred from the centrifuge bowl (with the aid of a rubber policeman) to 400-ml beakers in quantities not exceeding 2 cm in depth per beaker. This sediment was frozen and later freeze-dried before elemental analysis.

Elemental Analyses

Table 2 shows the elements on which analyses were obtained on the samples of grass hay, rumen fluid and rumen bacteria. With the exception of F, S, and Se, all elements were analyzed commercially.

In Vitro Rumen Fermentations

Exclusive of the inoculum preparation, the *in vitro* rumen fermentation technique utilized in these studies was a slight modification of that reported by Martinez and Church (1970).

Inoculum Preparation

Rumen fluid was collected and processed in a manner similar to that for elemental analysis, with the quantities of fluid varying

according to the amount of inoculum desired. After centrifuging the rumen liquor for 2 minutes at 1,500 rpm, the supernatant or clarified rumen fluid (CRF) was combined in the desired ratio with 39 degrees C, CO_2-saturated basal mineral mix (BMM) with the composition shown in Table 1 and 0.066 M, 39 degrees C, CO_2-saturated phosphate buffer (PB). The following inocula were used in the experiments reported herein: inoculum A = 50% CRF, 25% BMM and 25% PB; inoculum B = 33% CRF, 33% BMM and 33% PB; and inoculum C = 20% CRF, 80% BMM and 0% PB.

Table 1. Basal Mineral Medium

Compound	g/liter
$CaCl_2$	0.250
NaCl	2.000
KCl	2.000
$MgCl_2 \cdot 6H_2O$	0.250
$Na_2HPO_4 \cdot 7H_2O$	0.600
KH_2PO_4	0.300
$NaHCO_3$	1.750
Na_2SO_4	0.550
Urea	1.000
Glucose	0.250
Vitamin B_{12}	125 μg
Biotin	5 μg

Fermentation Conditions

Fermentations were conducted in 29 x 200 mm test tubes fitted with rubber stoppers with Bunsen valves. Each fermentation tube contained 50 mg of purified cellulose (Solka-Floc), 2 ml of the corresponding elemental treatments and 25 ml of the appropriate inoculum. Following inoculation, each fermentation tube was gassed with CO_2, stoppered and incubated in a 39 degrees C. water bath for 24 hours. Negative control tubes were frozen immediately after inoculation.

The initial pH of the inocula varied between 6. 3 and 6. 7 and was not adjusted to a constant value so as not to change its chemical composition from one trial to another.

Determination of Cellulose Digestion

Upon termination of the 24-hour incubation period, the contents of each fermentation tube were filtered, under suction, through a pre-weighed sintered glass crucible (50 ml capacity, porosity C). The crucibles with the residues were then dried in an oven at 90 degrees C. for 24 hours. The percent of cellulose digested was calculated with the formula:

$$\text{Percent cellulose digested} = \frac{K - C \times 100}{K}$$ where

C = undigested residue after fermentation and K = undigested residue with no fermentation (negative controls).

As mentioned in a previous report (Martinez and Church, 1970) this procedure does not measure cellulose digestion directly. However, there is a high correlation between this method and chemically determined cellulose.

Treatments and Design of Experiments

The elements Ca, Co, Cu, Fe, Mg, Mn, Mo, Ni, P, S and Zn and the compounds NaCl and KCl were tested in selected combinations for their effects upon in vitro rumen cellulose digestion (IVRCD). Solutions of these test elements and compounds were prepared so that addition of a total of 2 ml to the 25 ml inoculum yielded the desired final concentration in μg/ml.

Phosphorus was supplied as a combination of $Na_2HPO_4 \cdot 7H_2O$ and KH_2PO_4, S as Na_2SO_4 and Mo as $Na_2MoO_4 \cdot 2H_2O$, while the other test elements were supplied as their chloride salts unless otherwise indicated. The element or elements being tested were deleted from the basal mineral mix if present. However, the rumen fluid present in the inocula contributed certain amounts of each of the test elements--amounts which can be estimated by using the values presented in Table 2. Furthermore, procedures were designed to keep contamination of test and other elements to a minimum.

An experiment consisted of 2 duplicate trials--each trial with duplicate observations, that is, a total of 4 observations per treat-

ment. Time elapsed between each trial was never less than 3 days. The data were analyzed in either 4 x 4 or 4 x 4 x 4 factorial arrangements depending on whether 2 or 3 elements were tested. Treatment means were statistically compared with the "control" means by the use of the Least Significant Difference (LSD) procedure as described by Petersen (1967).

RESULTS AND DISCUSSION

Mineral Analyses

Table 2 shows the concentrations of 6 major and 15 trace elements in the grass hay (sole source of food), the clarified rumen fluid of the rumen bacteria of the fistulated steer that served as rumen fluid donor in all studies reported herein. Concerning the mineral composition of the grass hay, suffice to say that it appears to meet the maintenance needs of an adult steer. It should be pointed out, however, that the content of Co (0. 08 ppm), Se (0. 03 ppm) and Zn (22 ppm) in the dry matter may be considered marginal under some circumstances (Underwood, 1971).

Concentrations of all the analyzed elements in the clarified rumen fluid were considerably lower than those in the diet; the decrease ranging from 1. 5 times for Na to slightly more than 20 times for Mg and approximately 40 times for total S. Similarly, Evans and Davis (1966a) found that levels of inorganic P and S were much lower in the rumen fluid than in the diet, however, such fluid levels rose with dietary increments of the elements. Total S levels in the rumen fluid were reported to significantly increase with increases in dietary S (Upton and L'Estrange, 1971; Spais et al. 1968). Levels of intake and time of sampling after feeding resulted in signi-

ficant changes in the concentrations of dissolved Na, K, Ca, Mg and P in the rumen fluid (Fenner et al., 1969; Fenner and Damon, 1969). Poutiainen (1970) further indicated that a decrease in concentrations of Na and an increase in K, Ca, Mg and Cl occurred some 3 to 4 hours after feeding with P not being affected. Also his data showed a significant effect on levels of Na, K, Mg, Ca and Cl due to sampling points in the bovine rumen. Some relationships between concentrations of Na, K, Cl and inorganic P in ovine saliva and rumen fluid have been discussed by Bailey (1961). Poutiainen (1971) studied the input, flow and absorption of some major elements, while their passage from the reticulo-rumen was investigated by Lampila (1965). These and other suspected variables affecting the concentrations of major (and perhaps also of trace) minerals in the rumen fluid do not allow direct quantitative comparisons of data from various laboratories.

Rumen bacteria appear to concentrate major minerals to various degrees (Table 2). Bacterial levels of Na and P, for example, were approximately 6 times those in the feed, S about 5 times, K about 2 times, Ca 1. 3 times while Mg levels were the same. Information on major mineral composition of rumen microorganisms is not readily available.

As with the major minerals, concentrations of trace elements in clarified rumen fluid were much lower than those in the diet

Table 2. Mineral content of grass hay, rumen fluid and rumen bacteria.

Element	Grass hay	Rumen fluid	Rumen bacteria
Major elements	%	%	%
Ca	0. 66	0. 036	0. 88
K	0. 90	0. 120	2. 10
Mg	0. 29	0. 014	0. 29
Na	0. 54	0. 360	3. 60
P	0. 29	0. 036	1. 90
S	0. 16	0. 0042	0. 78
Minor elements	µg/g	µg/ml	µg/g
Al	46. 00	1. 30	265. 00
B	4. 30	0. 42	16. 00
Ba	26. 00	0. 70	37. 00
Cd	0. 10	0. 03	0. 50
Co	0. 08	<0. 01	3. 75
Cr	<3. 00	< 0. 15	<1. 50
Cu	12. 00	0. 83	22. 00
F	22. 00	1. 52	82. 00
Fe	66. 00	2. 00	525. 00
Mn	170. 00	2. 90	230. 00
Mo	1. 20	<0. 10	6. 20
Ni	2. 00	0. 02	7. 00
Se	0. 03	0. 0008	0. 50
Sr	22. 00	2. 30	46. 00
Zn	22. 00	0. 16	92. 00

(Table 2). Elemental analysis of strained rumen fluid by Sala (1957) yielded the following concentrations in ppm: Co, 0.06; Cu, 0.36; Fe, 2.25; Mn, 1.90; Mo, 0.076; and Zn, 1.17. For Fe, McNaught et al. (1950) reported values between 1 and 2 ppm in the fluid. Considering the differences in the composition of the diets, these values do not differ substantially from those on Table 2. To this effect, Zerebcov et al. (1971b) indicated that supplementation of certain trace elements (Co, Cu, Mn and Zn) resulted in increases in concentrations of these elements in the rumen, whereas, some changes in the concentrations and solubilities of Zn, Mn and Cu in the rumen, abomasum and small intestine of the ovine have been determined by Bremner (1970).

It appears that some elements influence the concentrations of others in the rumen fluid. Data presented by Mills (1960) suggested that Mo and S depressed the levels of soluble Cu in ovine rumen and abomasal fluids, however, such interaction was not corroborated by Evans and Davis (1966a). Nonetheless, Zerebcov et al. (1971b) found that supplementation with Cu, Mn or Zn resulted in an increase in Co in the rumens of bullocks.

Exclusive of Cr, rumen bacteria concentrated trace elements to various extents when compared to the diet. Cobalt was concentrated most extensively (47 times) and was followed by Se (16 times). Elements concentrated from 5 to 10 times included Al, Cd, Fe and

Mo, while B, Ba, Cu, F, Mn, Ni, and Sr were increased less than five-fold. In studies of Co in ovine rumen fluid, Tosic and Mitchell (1948) concluded that rumen microorganisms concentrated Co from their external environment to the extent that about 80 percent of the Co in the rumen contents was found in the microbes. Their data also showed that when hay containing 0. 07 ppm Co (in the dry matter) was fed to sheep, the rumen microbial fractions attained a level of 3. 62 ppm. These values are quite close to those found in this study. In a similar experiment Mitchell and Tosic (1949) reported that Cu, Ni, Mo and Zn were concentrated by ovine rumen microorganisms some 10 times, while V, Ti, Mn and Fe were concentrated to a lesser extent.

According to Whanger et al. (1970), rumen microorganisms (including protozoa and fine feed particles) from six-week-old lambs fed diets containing 0. 016 ppm Se in the dry matter concentrated the element two- to ten-fold. Yet, in a following experiment, Whanger (personal communication) found that rumen microbes (free of protozoa and fine feed particles) from six-week-old lambs fed diets varying in Se content concentrated the element approximately 18 times on the average. This value does not differ considerably from that obtained in this study (16 times), probably because the centrifuged microbial fractions were similar in composition.

Regarding the ash content of dried rumen bacteria, Ellis et al. (1958) found it to be 9.41 percent. This ash contained, in approximate ppm: Cu, 100; Co, trace; Fe, 300; Mo, 10; and Zn, 200. However, the dried, fat-free cells contained about 1 ppm of Mo.

It is appropriate to point out that some mineral contamination of the grass hay might have occurred during grinding for elemental analysis. Ammerman, Martin and Arrington (1970), for example, established that grinding citrus pulp in a standard Wiley mill significantly increased the concentrations of Fe, Zn, Cu, Mn and Na but had no effect on Ca, K, Mg and P. Also, a possible source of mineral contamination to the rumen bacteria was the metal bowl of the continuous-flow centrifuge, however, no attempt was made to substantiate this possibility.

In Vitro Rumen Fermentations

Mn and Zn

Table 3 shows the effects of Mn and Zn upon cellulose digestion when the inoculum consisted of 50% CRF, 25% BMM and 25% PB (inoculum A). The border means (main effects) indicate that 200 μg/ml added Mn and 50 μg/ml added Zn significantly reduced ($P < .05$) the amount of cellulose digested. Similarly, the within-table means (Mn x Zn means) show a significant decrease in cellulose digestion when 200 μg/ml of Mn and 50 μg/ml Zn were added.

Table 3. Effect of Mn and Zn on *in vitro* rumen cellulose digestion[a].

Mn added μg/ml	Zn added, μg/ml				Means (Mn)
	0	0.5	5.0	50	
	Percent cellulose digested[bc]				
0	68.00	65.91	61.69	33.28	57.22
2	67.68	67.22	61.82	38.11	58.71
20	64.76	61.39	63.37	45.25	58.69
200	39.74	41.85	43.65	42.59	41.96
Means (Zn)	60.05	59.09	57.63	39.81	

[a]Inoculum A (50% CRF, 25% BMM and 25% PB).

[b]Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c]Difference required for significance at P = .05 between control and treatment means: for within table means, LSD = 11.09; for border means (Mn, Zn), LSD = 5.54. See text for limitations on the use of the LSD test.

Of importance, however, is the significant ($P < .05$) interaction between Mn and Zn in which added Mn appears to partially protect against Zn toxicity. Addition of 50 μg/ml of Zn, in the absence of added Mn, significantly reduced the amount of cellulose digested to 33 percent, but addition of 20 μg/ml of Mn significantly increased digestion ($P < .05$) to about 45 percent. Two and 200 μg/ml added Mn also protected against Zn toxicity, but the increases were not statistically significant ($P > .05$).

Information on the individual effects of Mn or Zn upon rumen microbial activity is rather abundant, however, data indicating interaction of the two elements is lacking. Reports indicating the individual beneficial or toxic effects of Mn and/or Zn to rumen microbes under a variety of fermentation conditions include those of Chamberlain and Burroughs (1962), Robinson et al. (1960), Cunningham, Wise and Barrick (1966), Hubbert, Cheng and Burroughs (1958b), Little, Cheng and Burroughs (1958), Martinez and Church (1970), McNaught et al. (1950), Ott et al. (1964, 1966), Sala (1957), Woods (1965), Zerebcov and Nabiev (1971), and Zerebcov et al. (1971a, b). Reference has been made in the literature, however, to the counteracting effect of Mn upon Zn toxicity in some pure cultures of lactic acid bacteria (MacLeod and Snell, 1950). In a subsequent report, Tsuyuki and MacLeod (1951) found that Zn inhibited glycolysis in

Lactobacillus arabinosus and that such inhibition could be overcome by addition of Mn. These microorganisms were not of rumen origin.

The experiment summarized in Table 4 indicates that a change in the inoculum (A to B) had no appreciable effect on the Mn-Zn interaction. Additions of 20 and 200 μg/ml Mn, in the presence of 50 μg/ml added Zn, significantly ($P < .05$) increased cellulose digestion showing a protective effect of Mn against the toxicity caused by excessive Zn.

It is appropriate, at this time, to point out the limitations of the LSD test as used in this study. The LSD for within-table means (means in the factorial arrangement) is valid to compare only the control mean against the other means (3) within each row or within each column. For example, in Table 4 the means 65.29, 66.43, 68.53, and 56.25 are the control means for each of the corresponding rows; the other 3 means in each row can be compared only against the control mean. Similarly, 65.29, 65.10, 67.05, and 43.13 are the control means for each of their corresponding columns. Thus, since the LSD for within-table means is 6.60, it can be said that any of the three means in row 1 (0 μg/ml of added Mn) is significantly different ($P < .05$) from the control means if it is larger than 71.89 (65.29 + 6.60) or smaller than 58.29 (65.29 - 6.60). As far as the border means (main effect means) are concerned, the control mean is that which represents the addition of no elemental treatment; in

Table 4. Effect of Mn and Zn on in vitro rumen cellulose digestion[a].

Mn added μg/ml	Zn added, μg/ml				Means (Mn)
	0	0.5	5.0	50	
	Percent cellulose digested[bc]				
0	65.29	65.10	67.05	43.13	60.14
2	66.43	66.15	66.83	46.08	61.37
20	68.53	69.10	69.23	53.80	65.16
200	56.25	56.58	58.24	52.76	55.96
Means (Zn)	64.12	64.23	65.34	48.94	

[a] Inoculum B (33% CRF, 33% BMM and 33% PB).

[b] Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c] Difference required for significance at P = .05 between control and treatment means: for within table means, LSD = 6.60; for border means (Mn, Zn), LSD = 3.30. See text for limitations on the use of the LSD test.

Table 4 these are 64.12 and 60.14. Likewise, the other 3 main effect means can only be compared with the control mean. Comparisons other than those discussed above are not valid if the LSD test is used.

Ca and Zn

The effects of Ca and Zn upon *in vitro* rumen cellulose digestion are summarized in Table 5. Addition of 40 or 100 μg/ml Ca when 0 or 0.5 μg/ml Zn had been added to the medium resulted in a significant ($P < .05$) decrease in the amount of cellulose digested. When 5 or 50 μg/ml Zn were added with no added Ca, cellulose digestion was significantly reduced from the control by approximately 38 and 86 percent, respectively. However, additions of 20 or 40 μg/ml Ca significantly increased cellulose digestion by approximately 44 and 47 percent, respectively, when Zn was added at 5 μg/ml and increases of 168 and 379 percent, respectively, when Zn had been added at 50 μg/ml. Furthermore, additions of 100 μg/ml Ca resulted in a 386 percent increase in digestion of cellulose when Zn had been added at levels of 50 μg/ml. Thus, the data indicate the existence of a significant interaction between Ca and Zn, an interaction in which Ca partially protects against the inhibitory effects of excessive Zn. The border means show that Zn significantly depressed digestion at added levels of 5.0 and 50 μg/ml. The

Table 5. Effect of Ca and Zn on *in vitro* rumen cellulose digestion[a].

Ca added μg/ml	Zn added, μg/ml				Means (Ca)
	0	0.5	5.0	50	
	Percent cellulose digested[bc]				
0	62.25	62.40	39.03	8.38	43.01
20	60.70	62.17	56.04	22.48	50.35
40	54.74	53.66	57.26	40.11	51.44
100	40.31	37.30	38.05	40.70	39.09
Means (Zn)	54.50	53.88	47.59	27.92	

[a]Inoculum B (33% CRF, 33% BMM and 33% PB).

[b]Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c]Difference required for significance at P = .05 between control and treatment means: for within table means, LSD = 6.97; for border means (Ca, Zn), LSD = 3.48. See text for limitations on the use of the LSD test.

main effect of Ca, on the other hand, was stimulatory at added levels of 20 and 40 μg/ml and inhibitory at added levels of 100 μg/ml ($P < .05$).

Although information on the individual effects of Ca or Zn on rumen microbial activity is available (for Ca: Barth and Hansard, 1961; Bryant, Robinson and Chu, 1959; Burroughs et al., 1951; Davidson and Woods, 1959; Hubbert et al., 1958b; James, Street and Butcher, 1967; Jones, MacLeod and Blackwood, 1964), no reference is readily available to their interaction as was found in the present study. In experiments carried out with pure cultures of lactic acid bacteria (not of ruminal origin), MacLeod and Snell (1950) established that Ca, as well as Sr, Mn, and Mg ions, was able to relieve the growth inhibition caused by excessive Zn. However, Ca or Mg ions had no effect in overcoming the inhibition that excessive Zn ions had on glycolysis (Tsuyuki and MacLeod, 1951).

Mg and Ca

A summary of the effects of Mg and Ca upon cellulose digestion by mixed rumen microorganisms appears in Table 6. The border means show that the main effect of Mg was as follows: when compared to the control, 25 μg/ml added Mg significantly increased ($P < .05$) the amount of cellulose digested, 200 μg/ml significantly depressed digestion by approximately 30 percent and 50 μg/ml

Table 6. Effect of Mg and Ca on in vitro rumen cellulose digestion[a].

Mg added μg/ml	Ca added, μg/ml 0	40	100	200	Means (Mg)
	Percent cellulose digested[bc]				
0	75.10	77.50	72.50	41.29	66.60
25	69.99	77.27	70.66	60.17	69.52
50	67.10	64.40	75.41	62.02	67.23
200	47.24	43.06	44.75	52.33	46.84
Means (Ca)	64.86	65.56	65.83	53.95	

[a] Inoculum C (20% CRF and 80% BMM).

[b] Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c] Difference required for significance at P = .05 between control and treatment means: for within table means, LSD = 4.90; for border means (Mg, Ca), LSD = 2.45. See text for limitations on the use of the LSD test.

added Mg had no effect. The main effect of 40 and 100 μg/ml added Ca was non-significant, but addition of 200 μg/ml significantly ($P < .05$) depressed digestion, although not as much as 200 μg/ml added Mg.

Although several significant comparisons can be made between the within-table means (Mg x Ca means) only a few of interest are reported herewith. When no Ca was added to the incubation medium, additions of 25 or more μg/ml Mg significantly ($P < .05$) depressed cellulose digestion, however, the depression caused by 25 μg/ml added Mg was completely reversed by additions of 40 μg/ml Ca and that caused by adding 50 μg/ml Mg was totally reversed by adding 100 μg/ml Ca. The toxicity caused by adding 200 μg/ml Mg with 0, 40, or 100 μg/ml added Ca was partially reversed by additions of 200 μg/ml Ca. Similarly, in the absence of Mg, 200 μg/ml added Ca significantly depressed ($P < .05$) the amount of cellulose digested from 75 to 41 percent or by approximately 45 percent. This depression caused by 200 μg/ml added Ca was reversed partially, but significantly ($P < .05$), by additions of 25, 50 or 200 μg/ml Mg (from 41 percent to 60, 62, and 52 percent, respectively).

Uesaka *et al.* (1968) reported on an interaction between Ca and Mg which is very similar to that found in this study. In their *in vitro* rumen fermentations, they found that in a Mg-free medium

the addition of 800 μg/ml Ca depressed cellulose digestion, but the depression was alleviated by the addition of 50 to 400 μg/ml Mg. These Japanese workers also found significant interactions between Ca and P, P and Mg and Ca, P and Mg.

It is interesting to note in the previous experiment (Table 5), when total Mg was held constant at about 56 μg/ml of medium and no Zn was added, additions of only 40 μg/ml Ca significantly reduced cellulose digestion. In this experiment (Table 6), additions of 100 μg/ml Ca had no significant deleterious effect on cellulose digestion when total Mg levels were approximately 53 μg/ml (this corresponds to an added level of 25 μg/ml Mg; the difference being supplied by the rumen fluid). Since different inocula were used in each of the two experiments, the total Ca level, when 40 μg/ml Ca were added, was about 158 μg/ml in the experiment summarized in Table 5, while the total Ca level corresponding to addition of 100 μg/ml Ca (Table 6) was approximately 172 μg/ml. The discrepancy in the results between the two experiments can, perhaps, be attributed to the differences in the composition of the inoculum. Therefore, extreme care must be exercised when attempting comparisons between experiments.

Mn and Fe

The data in Table 7 represent the effects of Mn and Fe upon

Table 7. Effect of Mn and Fe on *in vitro* rumen cellulose digestion[a].

Mn added μg/ml	Fe added, μg/ml 0	1	10	100	Means (Mn)
	Percent cellulose digested[bc]				
0	66.12	69.43	72.57	63.67	67.94
2	69.27	71.40	71.09	62.87	68.66
20	67.12	71.13	72.06	54.74	66.26
200	34.24	35.30	47.03	26.29	35.72
Means (Fe)	59.18	61.81	65.69	51.89	

[a] Inoculum A (50% CRF, 25% BMM and 25% PB).

[b] Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c] Difference required for significance at P = .05 between control and treatment means: for within table means, LSD = 9.38; for border means (Mn, Fe), LSD = 4.69. See text for limitations on the use of the LSD test.

in vitro rumen cellulose digestion. The border means show that there was a significant increase ($P < .05$) in the amount of cellulose digested with addition of 10 μg/ml Fe. Addition of 100 μg/ml Fe or 200 μg/ml Mn significantly inhibited cellulose digestion. The addition of 200 μg/ml Mn, in the presence or absence of added Fe, proved inhibitory when the Mn x Fe means were compared. Of interest is the significant ($P < .05$) increase in digestion caused by the addition of 10 μg/ml Fe in the presence of 200 μg/ml Mn; indicating a partial protection by divalent Fe against the depression in cellulose digestion caused by excessive Mn.

Hubbert *et al.* (1958b), Little *et al.* (1958), Martinez and Church (1970), McNaught *et al.* (1950), Kawashima *et al.* (1969), Kim *et al.* (1969b) and Sala (1957) have studied various aspects of the Fe nutrition of rumen microorganisms under a variety of fermentation conditions. However, no mention has been made of a Mn-Fe interaction similar to that found in this study.

NaCl and KCl

Table 8 summarizes the effects of added NaCl and KCl upon cellulose digestion by rumen microorganisms. The border means indicate that the main effect of 0.1 percent added NaCl was to significantly ($P < .05$) stimulate cellulose digestion while 0.5 and 1.0 percent significantly depressed digestion by approximately 10 and 77

Table 8. Effect of NaCl and KCl on *in vitro* rumen cellulose digestion[a].

NaCl added %	KCl added, %				Means (NaCl)
	0	0.1	0.5	1.0	
	Percent cellulose digested[bc]				
0	34.67	50.39	57.30	38.13	45.12
0.1	49.28	59.04	54.56	49.02	52.98
0.5	61.01	57.60	43.46	0	40.52
1.0	25.39	15.85	1.00	0	10.56
Means (KCl)	42.59	45.72	39.08	21.79	

[a] Inoculum C (20% CRF and 80% BMM).

[b] Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c] Difference required for significance at P = .05 between control and treatment means: for within-table means, LSD = 3.78; for border means (NaCl, KCl), LSD = 1.89. See text for limitations on the use of the LSD test.

percent, respectively. A similar main effect was produced by additions of KCl; 0.1 percent significantly ($P < .05$) stimulated digestion while 0.5 and 1.0 percent depressed digestion. When no KCl was added or in the presence of 0.1 percent added KCl, additions of 0.1 or 0.5 percent NaCl significantly ($P < .05$) increased the amount of cellulose digested, but additions of 1.0 percent NaCl depressed digestion. Additions of 0.1 and 0.5 percent KCl significantly ($P < .05$) increased cellulose digestion and 1.0 percent had no effect on cellulose digestion when added NaCl levels were 0 and 0.1 percent. Additions of KCl, in the presence of 0.5 or 1.0 percent added NaCl, significantly depressed cellulose digestion with the higher concentrations of both compounds being quite toxic to the rumen microbes. However, it appears that additions of 0.1 percent NaCl were effective in counteracting the depression in digestion caused by 1.0 percent KCl.

Evidence of the involvement of the Na and K ions on rumen microbial activity has been presented by Bryant et al. (1959), Burroughs et al. (1951), Jones et al. (1964), and Lee and Matrone (1971). The effects of high salt intakes on in vivo and/or in vitro cellulose digestion have been reported by Cardon (1953), Elam (1961), and Kroger and Carroll (1964). Rumen fluid pH, in vitro microbial activity and rumen fluid levels of Na and K have been reported to increase as dietary K increased from 0.27 to 0.85 per-

cent (Devlin et al., 1969). Brink and Pfander (1961) indicated that 0.5 percent K appeared to be optimum for normal rumen function, while Khan et al. (1969) concluded that K in high concentrations (1,000 mM/liter) depressed glucose and urea utilization and VFA production as well as inhibiting other rumen microbial activities. Furthermore, Bergen (1970) demonstrated that any combination of cations or anions, normally present in the rumen, depressed in vitro cellulose digestion at a toxicity of 400 mOsm.

An interesting interaction between Na and K has been reported by Hubbert et al. (1958a). Their data revealed that K was essential for in vitro rumen cellulose digestion, but Na had no effect or depressed digestion in the absence of K. Also, additions of Na increased cellulose digestion only in the presence of K (100 to 400 μg/ml of medium). The data in this study (Table 8) show that both NaCl and KCl increased cellulose digestion, however, the control medium (no added NaCl or KCl) contained both Na and K derived from the rumen fluid and the phosphate salts in the basal mineral medium.

S, Mg and Ni

The effects of S, Mg and Ni upon cellulose digestion are shown in Table 9. Although a large number of significant comparisons between means can be made in a factorial arrangement of

Table 9. Effect of S, Mg and Ni on in vitro rumen cellulose digestion[a]

Mg added μg/ml	S added μg/ml	Ni added μg/ml 0	0.02	0.2	2.0	Means (Mg x S)
		Percent cellulose digested[bc]				
0	0	7.32	14.96	14.16	1.20	9.41
	10	68.63	73.93	72.36	6.98	55.47
	100	68.26	70.33	73.23	6.12	54.48
	1000	69.15	70.19	71.34	3.93	53.65
	Means (Ni x Mg)	53.34	57.36	57.77	4.56	
25	0	16.59	16.79	14.38	10.62	14.59
	10	71.75	71.03	73.99	39.36	64.03
	100	74.03	73.28	72.68	46.61	66.65
	1000	70.74	69.70	71.51	40.75	63.17
	Means (Ni x Mg)	58.27	57.70	58.14	34.33	
50	0	16.18	15.48	18.56	11.35	15.39
	10	63.99	70.31	66.44	45.73	61.62
	100	74.42	74.82	73.18	59.16	70.39
	1000	67.41	66.21	68.87	51.79	63.57
	Means (Ni x Mg)	55.50	56.70	56.76	42.01	
250	0	13.93	19.87	15.49	13.94	15.80
	10	61.85	57.01	61.47	47.64	57.01
	100	71.47	69.95	69.50	60.50	67.85
	1000	62.29	65.14	64.82	57.96	62.55
	Means (Ni x Mg)	52.38	53.01	52.82	45.01	
			Means (Ni x S)			
	0	13.50	16.77	15.65	9.27	
	10	66.55	68.09	68.56	34.93	
	100	72.04	72.09	72.15	43.09	
	1000	67.40	67.81	69.13	38.61	

[a] Inoculum C (20% CRF and 80% BMM).

[b] Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c] Difference required for significance at P = 0.5 between control and treatment means: for within table means, LSD = 13.98; for border means (Mg x S, Ni x Mg, Ni x S), LSD = 6.99. See text for limitations on the use of the LSD test.

this nature, only the main effects will be discussed here. The border means (Mg x S and Ni x S) clearly show that at least 10 μg/ml S should be added to this inoculum for maximum cellulose digestion. Added S at levels of 100 and 1000 μg/ml also stimulated cellulose digestion ($P < .05$) when compared to no S added regardless of the presence or absence of Ni and/or Mg. The Ni x Mg means denote the significant ($P < .05$) inhibition caused by 2 μg/ml of added Ni, however, such inhibition was partially reversed by additions of 25, 50, or 250 μg/ml Mg in the presence of at least 10 μg/ml added S. It appears that combinations of 50 or 250 μg/ml Mg and 100 or 1000 μg/ml S provided the best protection against the inhibition caused by addition of 2 μg/ml Ni. Added Mg, however, resulted in no increase in cellulose digestion when added Ni levels were 0.2 μg/ml or less and in the presence or absence of S.

The effects of S upon various aspects of rumen microbial activity have been studied extensively (Ammerman et al., 1963; Burroughs et al., 1951; Emery, Smith and Huffman, 1957; Emery, Smith and Faito, 1957; Halverson, Williams and Paulson, 1968; Hunt et al., 1954; Jusadasan and Thomas, 1971; Krabill, Alhassan and Satter, 1969; Martin et al., 1964; Mehren and Klett, 1971; Nader and Walker, 1970; Paulson, Baumann and Pope, 1968; Slyter et al., 1971; Streeter, Little and Mitchell, 1970; and Whanger and

Matrone, 1965. In a recent study Kennedy, Mitchell and Little, (1971) established a requirement of 1 to 2 μg/ml S from Na_2SO_4 in a simplified medium and 3 to 5 μg/ml in a complex medium for optimum starch digestion by washed suspensions of rumen microbes. Hubbert et al. (1958b) and Trenkle, Cheng and Burroughs (1958) reported that at least 10 μg/ml S were required for optimum cellulose digestion by washed suspensions, while Barton, Bull and Hemken (1971) have suggested a S concentration of 0.14 to 0.17 percent in the dry matter for optimum cellulose and lignocellulose digestion. Since the inoculum (A) used in this experiment contained 8.4 μg/ml S, it appears that this S concentration is not sufficient to support cellulose digestion in this type of fermentation.

Martinez and Church (1970) found no beneficial effects on cellulose digestion by washed suspensions of rumen microorganisms upon additions of Ni. Addition of 0.5 μg/ml Ni depressed digestion by 50 percent with no further decrease in digestion being caused by addition of levels up to 10 μg/ml.

The involvement of Mg in the activities of rumen microorganisms has been examined by several researchers (Ammerman et al., 1963, 1971; Bryant et al., 1959; Burroughs et al., 1951; James et al., 1967; Jones et al., 1964; Little et al., 1958; Martin et al., 1964. Although Chamberlain and Burroughs (1962) presented evidence of a Mg-Mn-F interaction in washed suspensions of rumen microbes

and Uesaka et al. (1968) demonstrated a Mg-Ca-P interaction affecting in vitro rumen cellulose digestion, no mention is made in the literature of a Mg-Ni or a Mg-S-Ni interaction affecting rumen microorganisms as was found in this study. There is however, a report by Abelson and Aldous (1950) indicating that the toxicity of Ni to Escherichia coli, Aerobacter aerogenes, Torulopsis utilis and Aspergillus niger (not usually rumen microorganisms) can be partially reversed by additions of excess Mg.

P, Mg and Ni

In this experiment (Table 10), S was held constant at added levels of 10 μg/ml and the concentrations of P, Mg and Ni were varied. Basically, the response in cellulose digestion to added Mg and Ni was similar to that of the prior experiment (Table 9). The Ni x Mg means indicate that 2.0 μg/ml added Ni, in the absence of Mg, practically stopped all microbial activity; but, in the presence of 25, 50 and 250 μg/ml added Mg, the inhibition was progressively reversed as Mg levels increased. The Ni and P means show a significant increase in the digestion of cellulose with additions of 1000 μg/ml of P. Similarly, the within-table means show that 1000 μg/ml of added P significantly ($P < .05$) increased cellulose digestion provided Mg was present. Thus, the data indicate a Ni-Mg interaction, where Mg partially reverses the inhibition caused by excess Ni and a Ni-Mg-P interaction--where P enhances the protection offered by Mg.

Table 10. Effect of P, Mg and Ni on in vitro rumen cellulose digestion[a]

Mg added μg/ml	P added μg/ml	Ni added μg/ml 0	0.02	0.2	2.0	Means (Mg x P)
		Percent cellulose digested[bc]				
0	0	67.88	67.47	65.47	2.76	50.89
	10	63.34	66.28	62.21	3.05	48.72
	100	67.58	66.64	66.24	5.56	51.51
	1000	70.81	68.94	67.97	6.43	53.54
	Means (Ni x Mg)	67.40	67.33	65.47	4.45	
25	0	66.76	69.65	66.80	18.35	55.39
	10	65.75	64.72	68.35	21.95	55.19
	100	68.15	62.29	65.96	25.60	55.50
	1000	65.53	63.50	66.70	38.33	58.51
	Means (Ni x Mg)	66.55	65.04	66.95	26.06	
50	0	65.02	60.04	63.59	37.33	56.49
	10	62.40	62.19	62.86	35.00	55.61
	100	69.66	66.52	66.91	41.32	61.10
	1000	65.90	66.01	67.21	45.54	61.16
	Means (Ni x Mg)	65.74	63.69	65.14	39.80	
250	0	66.70	67.29	67.61	48.82	62.61
	10	61.70	60.79	62.70	44.15	57.33
	100	69.47	68.11	68.89	44.68	62.79
	1000	62.04	61.52	60.78	55.89	60.06
	Means (Ni x Mg)	64.98	64.42	64.99	48.38	
			Means (Ni x P)			
	0	66.59	66.11	65.86	26.81	
	10	63.30	63.49	64.03	26.03	
	100	68.71	65.89	67.00	29.29	
	1000	66.07	64.99	65.66	36.54	

[a] Inoculum C (20% CRF and 80% BMM).

[b] Table means (within factorial arrangement) are means of four observations--two trials in duplicate.

[c] Difference required for significance at P = .05 between control and treatment means: for within table means, LSD = 6.77; for border means (Mg x P, Ni x Mg, Ni x S), LSD = 3.89. See text for limitations on the use of the LSD test.

Ammerman et al. (1965), Barth and Hansard (1961), Bryant et al. (1959), Burroughs et al. (1951), Chicco et al. (1965), Evans and Davis (1966a, b), Hall, Baxter and Hobbs (1961) and Raun et al. (1956), amongst others, have studied various aspects of P metabolism in rumen microorganisms. A P-Mg and Ca-P-Mg interaction affecting in vitro rumen cellulose digestion has been reported by Uesaka et al. (1968). Their data suggested that the P and Mg requirements of rumen bacteria increased as the Ca concentration in the medium increased, also, the Ca and Mg requirements seemed to increase as the P concentration was increased. The P-Mg interaction was not apparent with the type of inoculum and the elemental levels tested in the present study.

S, Mg and Co

The border means, Mg x S and Co x S, clearly indicate that additions of S (at least 10 μg/ml) are necessary for optimum cellulose digestion (Table 11). These results are in accordance with those obtained in an experiment that has been discussed previously (Table 9). Additions of 2.5 and 5.0 μg/ml Co, in the absence or presence of 25, 50 and 250 μg/ml added Mg (excepting the combination of 2.5 μg/ml Co and 250 μg/ml Mg) significantly depressed ($P < .05$) cellulose digestion. However, additions of Mg (Co x Mg means) partially but significantly ($P < .05$) reversed the inhibition

Table 11. Effect of S, Mg and Co on in vitro rumen cellulose digestion[a]

Mg added, μg/ml	S added, μg/ml	Co added μg/ml 0	0.5	2.5	5.0	Means (Mg x S)
		Percent cellulose digested[bc]				
0	0	25.48	22.64	24.34	16.33	22.20
	10	70.87	69.41	45.25	15.92	50.36
	100	67.61	69.28	41.59	20.25	49.68
	1000	69.44	66.28	47.20	25.44	52.09
	Means (Co x Mg)	58.35	56.90	39.59	19.49	
25	0	21.55	27.55	26.35	19.06	23.63
	10	72.58	71.90	56.87	33.37	58.68
	100	70.66	71.16	55.41	35.11	58.08
	1000	69.80	67.99	49.80	32.71	55.07
	Means (Co x Mg)	58.65	59.65	47.10	30.06	
50	0	23.70	28.42	28.37	22.85	25.83
	10	70.00	70.71	64.21	46.04	62.74
	100	71.47	72.53	60.75	46.52	62.82
	1000	69.69	63.99	54.58	39.43	56.90
	Means (Co x Mg)	58.71	58.91	51.95	38.71	
250	0	24.78	25.19	25.31	22.21	24.37
	10	65.69	67.84	60.32	39.90	58.44
	100	65.68	68.53	58.80	44.87	59.47
	1000	60.29	55.95	48.92	35.37	50.13
	Means (Co x Mg)	54.11	54.37	48.34	35.58	
			Means (Co x S)			
	0	23.88	25.95	26.09	20.11	
	10	69.78	69.96	56.66	33.81	
	100	68.85	70.37	54.13	36.69	
	1000	67.30	63.55	50.10	33.24	

[a] Inoculum C (20% CRF and 80% BMM).

[b] Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c] Difference required for significance at P = .05 between control and treatment means: for within table means, LSD = 11.62; for border means (Mg x S, Co x Mg, Co x S), LSD = 5.81. See text for limitations on the use of the LSD test.

caused by added Co. When comparing within-table means (LSD = 11.62), it is apparent that additions of 50 or 250 μg/ml Mg in the presence of 10 or 100 μg/ml added S completely protected against the inhibition caused by additions of 2.5 μg/ml Co. It appears that, in order for Mg to be effective in protecting against the toxicity of excessive Co, S has to be present in adequate amounts so as to otherwise support maximum cellulose digestion.

Although a similar protective action of Mg against the inhibition caused by excessive Co or Ni in some non-rumen microorganisms (Escherichia coli, Aerobacter aerogenes, Torulopsis utilis and Aspergillus niger) has been reported by Abelson and Aldous (1950), and in E. coli, B. magaterium and A. aerogenes by Webb (1970a, b), no information is available on this interaction as it affects rumen microbes.

Since vitamin B_{12} is synthesized by the rumen microorganisms and since Co is an integral part of this vitamin, the effects of Co on rumen microbial activities have probably been studied to a greater extent than any other trace element. Apparently, when concentrations of Co in the rumen contents of sheep, drop below a concentration of 40 mμg Co/g (20μμg/ml of the bacteria-free supernatant), synthesis of vitamin B_{12} falls drastically (Marston et al., 1961). Also, Co-deficient rations caused significant decreases in the numbers of bacteria as well as discernible morphological changes (Gall

et al., 1949). Studies with a variety of inocula and under various fermentation conditions indicate that, on the average, additions of more than 12 ppm Co to the incubation medium results in depressed microbial activity (Hubbert et al., 1958b; Little et al., 1958; Martinez and Church, 1970; McNaught et al., 1950; Sala, 1957; Salsbury, Smith and Huffman, 1956). In contrast, Streeter (1961) found that levels ranging between 10 and 50 ppm added Co proved stimulatory to cellulose digestion. Other aspects of the involvement of Co in rumen microbial activities have been examined by Johnson, Bentley and Moxon (1956), Jones et al. (1964), Kawashima et al. (1969), Kim et al. (1969b), Mitchell and Tosic (1949), Tosic and Mitchell (1948) and Zerebcov et al. (1971b).

It is interesting to note that when Zerebcov et al. (1971a) added Co to the rumens of bullocks to make a final concentration of 2.95 mg/liter, some synthetic processes in the rumen were inhibited. This in vivo inhibitory value (2.95 mg/liter) is not far from the in vitro inhibitory value (2.5 μg/ml added Co) found in this study.

P, Mg and Co

The experiment summarized in Table 12 was designed to test the effects of P (with S being kept constant) upon the previously demonstrated Mg-Co interaction (Table 11). The Co x Mg means indicate a significant reduction ($P < .05$) in the amount of cellulose

Table 12. Effect of P, Mg and Co on *in vitro* rumen cellulose digestion[a]

Mg added µg/ml	P added µg/ml	Co added µg/ml 0	0.5	2.5	5.0	Means (Mg x P)
		Percent cellulose digested[bc]				
0	0	72.13	68.93	46.64	26.39	53.52
	10	70.27	72.28	46.43	17.08	51.51
	100	67.43	67.21	46.22	16.13	49.25
	1000	64.96	64.47	49.90	17.81	49.28
	Means (Co x Mg)	68.70	68.22	47.30	19.35	
25	0	71.06	73.25	69.53	50.68	66.13
	10	72.10	74.65	66.61	43.17	64.13
	100	66.33	64.74	61.02	38.39	57.62
	1000	62.87	59.36	53.46	45.19	55.22
	Means (Co x Mg)	68.09	67.99	62.65	44.36	
50	0	70.54	73.34	72.19	58.71	68.69
	10	73.14	70.94	70.43	55.32	67.46
	100	60.16	61.69	59.42	51.70	58.24
	1000	61.18	55.84	53.22	42.45	53.20
	Means (Co x Mg)	66.25	65.45	63.81	52.07	
250	0	65.31	67.03	66.71	53.40	63.11
	10	65.86	64.99	65.02	55.05	62.73
	100	53.92	47.78	50.06	46.66	49.60
	1000	54.71	43.15	42.43	39.74	45.00
	Means (Co x Mg)	59.95	55.73	56.05	48.71	
		Means (Co x P)				
	0	69.76	70.64	63.77	47.29	
	10	70.34	70.71	62.12	42.65	
	100	61.96	60.35	54.18	38.22	
	1000	60.93	55.70	49.75	36.32	

[a] Inoculum C (20% CRF, 80% BMM).

[b] Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c] Difference required for significance at P = .05 between control and treatment means: for within table means, LSD = 21.26; for border means (Mg x P, Co x Mg, Co x P), LSD = 10.63. See text for limitations on the use of the LSD test.

digested with additions of 2.5 or 5.0 μg/ml Co in the absence of added Mg. Additions of 25 and 50 μg/ml Mg, however, significantly ($P < .05$) reversed the toxicity of 2.5 μg/ml added Co; whereas, additions of 25, 50 and 250 μg/ml Mg significantly reversed the inhibition caused by additions of 5.0 μg/ml Co. The Mg x P and Co x P means show that P offered no protection against the inhibition resulting from additions of excessive Co. In addition, it appears that added P, in concentrations of 100 to 1000 μg/ml, tended to decrease the efficiency of Mg in reversing the inhibition caused by excess Co as demonstrated by the within-table means.

Mg, Co and Ni

The protective effect of Mg against the individual inhibition of excessive Ni or excessive Co has been established (Tables 9, 10, 11 and 12). It seemed plausible, therefore, to test this protective effect when both elements--Ni and Co--were added to the incubation medium in the presence of constant levels of S and P. From the data summarized in Table 13, it is clear that, in the absence of added Mg and Co, 2.0 μg/ml added Ni significantly depressed digestion ($P < .05$). Also, in the absence of Mg and Ni, 2.5 μg/ml added Co significantly reduced digestion. These results are in agreement with those in Tables 9, 10, 11 and 12. Also, corroborating previous results is the fact that in the absence of added Ni, additions

Table 13. Effect of Mg, Co, and Ni on in vitro rumen cellulose digestion[a]

Co added μg/ml	Mg added μg/ml	Ni added μg/ml 0	0.02	0.2	2.0	Means (Co x Mg)
		Percent cellulose digested[bc]				
0	0	57.94	58.36	61.22	1.03	44.64
	25	60.23	56.04	57.05	5.83	44.79
	50	60.14	58.23	61.84	27.71	51.98
	250	59.89	55.71	55.42	37.01	52.01
	Means (Ni x Co)	59.55	57.08	58.88	17.90	
0.5	0	61.39	59.08	58.47	0.65	44.90
	25	59.93	57.94	56.09	9.35	45.83
	50	59.65	56.22	55.71	27.01	50.09
	250	56.71	56.61	55.15	37.55	51.50
	Means (Ni x Co)	59.88	57.46	56.35	18.64	
2.5	0	27.15	11.14	15.75	0,70	13.69
	25	32.53	35.00	34.12	5.39	26.76
	50	46.74	43.69	41.06	11.94	35.86
	250	42.72	43.61	42.53	29.23	39.52
	Means (Ni x Co)	37.29	33.36	33.37	11.81	
5.0	0	6.74	7.51	7.26	1.89	5.85
	25	10.05	17.31	8.67	2.71	9.68
	50	21.61	21.02	23.55	5.60	17.95
	250	29.13	28.84	25.63	9.48	23.27
	Means (Ni x Co)	16.88	18.67	16.28	4.92	
		Means (Ni x Mg)				
	0	38.31	34.02	35.67	1.07	
	25	40.68	41.57	38.98	5.82	
	50	47.49	44.79	45.54	18.06	
	250	47.11	46.20	44.68	28.32	

[a] Inoculum C (20% CRF, 80% BMM).

[b] Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c] Difference required for significance at P = .05 between control and treatment means: for within table means, LSD = 8.52; for border means (Co x Mg, Ni x Co, Ni x Mg), LSD = 4.26. See text for limitations on the use of the LSD test.

of Mg partially arrested the inhibition caused by additions of 2.5 and 5.0 μg/ml Co; and in the absence of added Co, additions of Mg partially overcame the depression in cellulose digestion caused by additions of 2.0 μg/ml Ni. As the levels of added Ni and Co, singly or in combinations, reached 0.2 and 2.5 μg/ml, respectively, the inhibition of cellulose digestion progressively increased. Nonetheless, the presence of added Mg ameliorated the depression in cellulose digestion caused by the combination of Ni and Co. Thus, the protection offered by additions of Mg against the inhibition in cellulose digestion by rumen microbes resulting from excesses of Ni or Co or combinations of both has been reaffirmed.

Some implications of the interactions thus far found in this study (Mg-Ni, Mg-Co, Mg-Ni-P, Mn-Zn, Ca-Zn, Mg-Ca, Mn-Fe and NaCl-KCl) include the avoidance of indiscriminate additions of some minerals to incubation media for *in vitro* rumen fermentation, while neglecting others. The same can, perhaps, be said for *in vivo* situations. A specific circumstance, for example, is the use of intraruminal Co bullets. If the long-term release of Co from said bullets is extensive, concentrations of this element in the rumen may reach levels which can be inhibitory to some synthetic rumen processes particularly if Mg is limiting (Zerebcov *et al.*, 1971a, reported that Co levels in the rumen of only 2.95 mg/liter inhibited some synthetic processes).

S, Mo and Cu

The data in Table 14 illustrate the effects of S, Mo and Cu and their combinations upon cellulose digestion by rumen microorganisms. In the absence of Cu toxicity (0.5 μg/ml or more added Cu), additions of at least 10 μg/ml S were necessary for optimum cellulose digestion. Thus, the S requirement has again been confirmed. The Cu x Mo means show that, for all practical purposes, additions of 0.5 and 1.0 μg/ml Cu stopped microbial activity as measured by the amount of cellulose digested. It appears then, that in the presence of S and in the absence of added Cu, 1000 μg/ml added Mo significantly ($P < .05$) inhibited cellulose digestion. In the presence of S and 0.1 μg/ml added Cu, the inhibitory level ($P < .05$) of added Mo was reduced to 100 μg/ml. However, additions of 10 to 1000 μg/ml Mo significantly ($P < .05$) increased cellulose digestion when S and Cu were not added, but in the presence of added Cu (0.5 μg/ml) this stimulation did not occur. Thus, there seems to be no evidence of the effectiveness of either S or Mo or both in arresting the inhibition caused by excessive Cu.

Martinez and Church (1970) incubated washed suspensions of rumen microorganisms with graded levels of added Mo. Their results indicate that 10 to 100 μg/ml added Mo significantly stimulated cellulose digestion, while 500 μg/ml had no effect. Similarly, Uesaka, Kawashima and Zembayashi (1965) indicated acceleration

Table 14. Effect of S, Mo, and Cu on in vitro rumen cellulose digestion[a]

Mo added μg/ml	S added μg/ml	Cu added μg/ml 0	0.1	0.5	1.0	Means (Mo x S)
		Percent cellulose digested[bc]				
0	0	14.71	18.16	14.74	2.88	12.62
	10	61.71	58.86	16.87	2.83	35.07
	100	62.12	61.83	8.49	0.00	33.11
	1000	61.97	60.43	7.99	0.00	32.60
	Means (Cu x Mo)	50.13	49.82	12.02	1.43	
10	0	23.22	22.01	7.89	2.99	14.03
	10	59.43	58.56	11.36	4.63	33.49
	100	63.36	57.72	3.70	0.00	31.19
	1000	61.21	59.50	5.91	0.00	31.65
	Means (Cu x Mo)	51.80	49.45	7.21	1.90	
100	0	24.91	23.31	5.59	4.29	14.53
	10	56.90	50.05	5.99	3.78	29.17
	100	58.66	52.65	2.50	0.00	28.45
	1000	55.81	52.62	2.98	0.00	27.85
	Means (Cu x Mo)	49.07	44.66	4.27	2.02	
1000	0	30.03	15.91	4.97	3.77	13.67
	10	54.62	29.58	2.59	5.07	22.96
	100	49.24	33.10	0.00	0.00	20.58
	1000	38.63	27.05	0.00	0.00	16.42
	Means (Cu x Mo)	43.13	26.41	1.89	2.21	
			Means (Cu x S)			
	0	23.22	19.85	8.30	3.48	
	10	59.16	49.26	9.20	4.08	
	100	58.34	51.33	3.67	0.00	
	1000	54.40	49.90	4.22	0.00	

[a]Inoculum C (20% CRF and 80% BMM).

[b]Table means (within the factorial arrangement) are means of four observations--two trials in duplicate.

[c]Difference required for significance at P = .05 between control and treatment means: for within table means, LSD = 6.60; for border means (Mo x S, Cu x Mo, Cu x S), LSD = 3.30. See text for limitations on the use of the LSD test.

of cellulose digestion by additions of Mo, with the element being toxic at levels of 2, 500 μg/ml. McNaught et al. (1950) reported that rumen bacteria tolerated between 100 and 1000 ppm Mo but 2000 ppm were definitely inhibitory. In contrast, Sala (1957) found Mo to be toxic to rumen microbes when added in concentrations of 20 ppm or more. In vivo trials carried out by Varela, Escriba and Boza (1970) showed a significant increase in the digestibilities of fiber with additions of Mo to the diet.

Additions of Cu to in vitro rumen fermentations have not proven beneficial to cellulose digestion with very low concentrations of the element being toxic to the rumen microbes (Martinez and Church, 1970; McNaught et al. 1950; Sala, 1957). Copper has been found, however, to stimulate rumen bacterial synthesis (Uesaka et al., 1965; Zerebcov et al., 1971a). Various aspects of the effects of Cu or Mo or both upon rumen microbial activity have been reported by Evans and Davis (1966a), Davis et al. (1956), Kawashima et al. (1969), Kim et al. (1969a, b), Slyter and Wolin (1967), Spais et al. (1968) and Zerebcov et al. (1971b).

Concerning the interactions of S, Mo, Cu and P, Evans and Davis (1966b) concluded that the depression caused in cellulose digestion by Cu was lessened by added S. This was not apparent in the present study. Ellis et al. (1958) suggested a possible relationship between Cu and Mo since the individual addition of each

did not stimulate cellulose digestion, but the addition of both resulted in a significant additive effect. Again, this Cu-Mo interaction was not apparent in the data presented in Table 14. In contrast, the results of this study are in closer agreement with those of Kawashima et al. (1968) in which the addition of Mo in concentrations of 100 to 1000 μg/ml and in the presence of S, significantly depressed at least one phase of rumen bacterial activity. Furthermore, Zembayashi et al. (1968) investigated the effects of Cu, Mo, S and P upon volatile fatty acid (VFA) production by rumen protozoa with their conclusions being quite similar to those of this study. Their data showed that additions of Cu to the medium did not alleviate the depression caused by excessive Mo, whereas, additions of Cu (0.5 μg/ml) tended to promote the inhibition of excessive Mo; additions of S aggravated the toxicity of excessive Mo; and the inhibitions caused by additions of both Mo and S could not be alleviated by adding Cu to the incubation medium.

From the results obtained in this study and those of the Japanese investigators it appears that Cu, Mo and S affect cellulose digestion by rumen bacteria and VFA production by rumen protozoa in a similar manner. Also, it appears that the interaction of Cu-Mo-S affecting rumen microorganisms, both bacteria and protozoa, is not similar to that which has been established for the host animal.

SUMMARY AND CONCLUSIONS

A series of factorial experiments (4 x 4 and 4 x 4 x 4) was designed to investigate the effects of selected combinations of some major and trace elements upon in vitro rumen cellulose digestion. Inocula, varying in ratios of clarified rumen fluid (strained through No. 50 cheesecloth and centrifuged at 365 x g for 2 minutes) to basal mineral medium to phosphate buffer, were incubated at 39 degrees C. with purified cellulose and the corresponding treatments of test elements. After a 24-hour incubation period, cellulose digestion was calculated. In addition, the mineral composition of rumen fluid and rumen bacteria was studied in relation to the mineral composition of the diet of a fistulated steer.

Mineral analysis for 6 major and 15 trace elements clearly indicated that, when compared to the diet, concentrations of these elements in the clarified rumen fluid were several times lower, whereas concentrations in rumen bacteria were several times higher. In general, rumen bacterial appeared to concentrate trace elements to a greater extent than major elements with approximately the following overall distribution: elements concentrated in excess of 20 times included Co; 10 to 20 times--Se; 5 to 10 times--Na, P, Al, Fe, Mo; 1 to 5 times--Ca, K, Mg, S, B, Ba, Cd, Cu, F, Mn, Ni, Sr, and Zn. Chromium was the only element found to be in les-

ser concentrations in rumen bacteria than in the diet.

Significant interactions ($P < .05$) affecting in vitro rumen cellulose digestion were found amongst the following combinations of elements: Mn-Zn, Ca-Zn, Mg-Ca, Mn-Fe, S-Mg-Ni, P-Mg-Ni, Mg-Co, P-Mg-Co, Mg-Co-Ni, and Cu-Mo-S. Additions of non-toxic levels of Mn partially but significantly reversed the inhibition in cellulose digestion caused by additions of excessive Zn. This protective effect remained significant when ratios of clarified rumen fluid to basal mineral medium to phosphate buffer were altered. Similarly, additions of Ca to the incubation medium significantly ($P < .05$) protected against the toxicity of excessive Zn. The significant ($P < .05$) depression caused by excessive Ca, on the other hand, was partially but significantly reversed by additions of Mg. Added Fe (only at levels of 10 μg/ml) had some protection against the depression in cellulose digestion caused by excessive Mn.

Additions of 0.1 percent NaCl were effective in counteracting the depression in cellulose digestion caused by excesses of KCl, however, additions of larger quantities of NaCl in the presence of 1.0 percent KCl resulted in total suppression of cellulose digestion.

Significant interactions ($P < .05$) were found between S, Mg and Ni. Additions of non-toxic levels of Mg partially reversed the severe depression in digestion caused by excessive Ni with additions of S or P significantly enhancing this protection. Non-toxic levels

of Mg were also successful in alleviating the depressing effects of excessive Co. Additions of S or P, however, were ineffective in augmenting the protection offered by Mg. When Mg, Co and Ni were tested simultaneously, Mg offered the already established protection against Ni and Co individually as well as against combinations of the two elements. However, as the concentrations of Ni and Co increased, the depression in cellulose digestion also increased, but the protection offered by Mg decreased.

Copper was found to be highly toxic to rumen microorganisms (additions of 0.5 μg/ml practically stopped all microbial activity). Neither Mo nor S or both were effective in alleviating the depression caused by excessive Cu. In lieu, the simultaneous presence of the three elements, in concentrations in which individually were non-deleterious to cellulose digestion, proved toxic to cellulose digestion. Nonetheless, additions of Mo significantly ($P < .05$) increased digestion in the absence of added Cu and S.

A S requirement by rumen microorganisms for optimum cellulose digestion was established in three separate experiments. A level of 8.4 μg/ml S--present in the inoculum--did not support adequate cellulose digestion, whereas additions of 10 μg/ml S to the inoculum resulted in maximum digestion.

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APPENDIX

Appendix Table 1. Sample analysis of variance for a 4 x 4 factorial (Ca and Mg)

Source	DF	SS	MS	F
Trial	1	32.8615	32.8615	
Ca	3	1684.7986	528.2662	49.94**
Mg	3	5336.7961	1778.9320	168.18**
Trial x Ca	3	24.2389	8.0796	0.76
Trial x Mg	3	67.1591	22.3864	2.12
Ca x Mg	9	3081.7475	342.4166	32.37**
Trial x Ca x Mg	9	67.2644	7.4738	0.71
Error[a]	15	158.6624	10.5774	

** $P < .01$

[a]The trial effect was removed from the MSE.

Appendix Table 2. Sample analysis of variance for a 4 x 4 x 4 factorial (Ni x Co x Mg)

Source	DF	SS	MS	F
Trial	1	3203.5599	3203.5599	
Ni	3	39902.0617	13300.6872	366.27**
Co	3	52424.4608	17474.8202	481.21**
Mg	3	8269.0661	2756.3554	75.90**
Trial x Ni	3	157.9415	52.6472	1.45
Trial x Co	3	64.3676	21.4559	0.59
Trial x Mg	3	489.4201	166.1400	4.58
Ni x Co	9	6852.1424	761.3492	20.97**
Ni x Mg	9	2494.2599	277.1400	7.63**
Co x Mg	9	2413.6435	268.1826	7.39**
Trial x Ni x Co	9	393.0537	43.6726	1.20
Trial x Ni x Mg	9	139.3557	15.4840	0.42
Trial x Co x Mg	9	444.8466	49.4274	1.36
Ni x Co x Mg	27	4987.8125	184.7337	5.09**
Trial x Ni x Co x Mg	27	589.8084	21.8447	0.60
Error[a]	63	2287.7936	36.3141	

** $P < .01$

[a]The trial effect was removed from the MSE.