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EFFECT OF DIVALENT IONS ON RUMINAL ENZYME ACTIVITIES IN SHEEP

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The effect of different salts of divalent ions as potential inhibitors and stimulators of ruminal alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) and glutamate dehydrogenase (GDH) activities in sheep was assessed using biochemical assays. Ruminal fluid was obtained from six cannulated ewes. Effects of $ZnCl_2$, $CuCl_2$, $MgCl_2$, $BaCl_2$ and $CoCl_2$ on ruminal enzyme activities were tested after incubation of ruminal fluid in the presence of the salts at 37°C for 30 min. Two different concentrations of salts of divalent ions were tested (5 and 20 $x10^{-3}$ mol/L). The higher concentration of Cu^{2+} and Zn^{2+} produced a higher inhibitory effect (from 50 to 70% of control ALT and AST activity) by Cu^{2+} and Zn^{2+}. The lower assayed concentration of Cu^{2+} produced 57% inhibition of GDH activity. Salts of divalent ions such as Ba^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and Mg^{2+} resulted in the activation of GDH from 216 to 297% of total GDH activity, when assayed at a relative concentration of 20 x 10^{-3} mol/L. The lower concentration of Ca^{2+} and Zn^{2+} iduced activation of ALT from 146 to 186%, while Cd^{2+}, Zn^{2+} and Ca^{2+} resulted in the activation of AST ranging from 127 to 172%. The results of the study indicate that the effect of divalent ions on ruminal enzyme activities depend on their relative concentration and which enzyme is assayed.

Key words: divalent ion, enzyme activation, enzyme inhibition, ruminal enzyme, sheep

INTRODUCTION

The role of the gastrointestinal tract and associated microflora in the digestion and metabolism of nitrogenous feed components is well established.

In particular, the effect of diet composition on ammonia and urea production and metabolism has received considerable attention (Parker *et al.,* 1995).

Ammonia is the main precursor for microbial protein synthesis in the rumen and in order for optimal fermentation to occur it must be present in excess of microbial requirements. Ammonia assimilation by rumen microbes depends on

many factors such as rumen pH (De Veth *et al.,* 1999), rumen ammonia concentration (Pal *et al.,* 1998) and rumen ammonia-assimilating activity.

De Veth *et al.* (1999) reported that rumen digestibility of pasture dry matter was optimized at pH 6.35 and synthesis of microbial protein was optimized at pH 6.13.

Many papers have dealt with the study of the effectiveness of various inhibitors on the activity of rumen urease. Reducing the rate of ammonia nitrogen release from dietary urea would increase its utilization in ruminants both *in vivo* (Musalia *et al.,* 2000, Puga *et al.,* 2001, Zhang *et al.,* 2002) and *in vitro* (Ludden *et al.,* 2000a). Ruminal microflora may often be capable of adapting to chronic administration of urease inhibitors, thereby limiting its practical use in improving the utilization of dietary urea *in vitro* (Ludden *et al.,* 2000b).

Several ammonia-assimilation reactions by ruminal bacteria are known. In *Ruminococcus flavefaciens* and *Prevotella ruminicola* glutamate dehydrogenase (GDH) appears to be the predominant route of ammonia assimilation irrespective of ammonia concentration, and peptides modulate GDH activity in *P. ruminicola* (Kirk *et al.,* 2000).

The aim of our study was to determine the effect of several divalent ions on ruminal enzyme activities of sheep *in vitro*.

MATERIAL AND METHODS

Animals

Six adult, cannulated, non-lacting female sheep (*Ovis aries*) weighing between 30 to 35 kg were used. The animals were housed individually, fed twice daily with a diet based on dehydrated alfalfa and milled corn grain in a 3:1 DM ratio, vitamin and mineral supplement and free access to water. EU requirements related to laboratory animal welfare were met.

Ruminal fluid collection

Ruminal fluid was obtained by means of a silicone tube attached to a vacuum source through a ruminal cannula 2 hours after morning feeding. The ruminal fluid was strained through four layers of cheesecloth within 30 minutes of collection.

Protocol

Effect of $ZnCl_2$, $CuCl_2$, $MgCl_2$, $CdCl_2$, $BaCl_2$ and $CoCl_2$ on ruminal enzyme activities was tested by incubation of ruminal fluid in the presence of different salts of divalent ions at 37°C for 30 min. Two different concentrations of salts were tested (5 and 20 x 10^{-3} mol/L).

Ruminal alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) and glutamate dehydrogenase activities (GDH) were determined by the colorimetric method using spectrophotometric kits.

Effect (activation/inhibition) was expressed as a percentage, considering 100% activity obtained when ruminal fluids were incubated without salts of divalent ions.

Statistical analysis

Statistical significance of the differences between values was determined by paired Student's *t*-test.

Chemicals

Magnesium chloride, cupri chloride, cobalt chloride, cadmium chloride, barium chloride and zinc chloride were obtained from SIGMA-Aldrich Chemie (Steinheim, Germany). Kits for alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase assays were obtained from LACHEMA (Brno, Czech Republic). Kits for glutamate dehydrogenase assay were purchased from RANDOX Laboratories (Crumlin, United Kingdom).

RESULTS

The effect of different salts of divalent ions when assayed at a concentration of 5 x 10^{-3} mol/L on enzyme activities of ruminal fluid is shown in Table 1.

Table 1. Effect of different divalent salts when assayed at a relative concentration of 5 x 10^{-3} mol/L on enzyme activities of ruminal fluid

Salt	ALT	AST	GGT	GDH
$ZnCl_2$	186.6± 5.9***	157.8±10.3**	72.1±8.2*	168.4±7.3***
$BaCl_2$	120.5±11.5 NS	114.3±5.8 NS	91.5±7.3 NS	86.1±8.7 NS
$CoCl_2$	146.6±5.1***	172.6±6.5***	323.6±21.6***	327.6±7.5***
$CdCl_2$	124.6±8.5*	127.1±7.1*	89.1±5.0 NS	106.0±15.9 NS
$MgCl_2$	107.4±5.0 NS	110.1±4.8 NS	131.6±12.3 NS	156.1±11.7**
$CuCl_2$	104.3±7.7 NS	122.4±8.6 NS	118.3±6.7*	43.9±6.0**

Results are expressed as percentage of ruminal enzyme activity, considering 100% activity obtained when ruminal fluid was incubated without divalent salts. Data are mean of six determinations ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 and NS not significant

The lower assayed concentration (5 x 10^{-3} mol/L) produced 57% inhibition of GDH activity by Cu^{2+}. The same concentration assayed, however, produced a high activatory effect (from 146 to 186% of total ALT activity) by Co^{2+} and Zn^{2+}. Cadmium, zinc and cobalt produced activation of AST ranging from 127 to 172%.

Negligible activation of total ALT and AST activities was detected when Mg^{2+} and Cu^{2+} were assayed. Barium was the only divalent ion assayed that produced negligible either activation or inhibition of all enzymes tested (ALT, AST, GGT and GDH).

The effect of different salts of divalent ions when assayed at a relative concentration of 20 x 10^{-3}mol/L on enzyme activities of ruminal fluid is shown in Table 2.

Table 2. Effect of different divalent salts when assayed at a relative concentration of 20 x 10^{-3} mol/L on enzyme activities of ruminal fluid

Salt	ALT	AST	GGT	GDH
$ZnCl_2$	43.3±3.2***	73.6±2.6*	96.6±0.02 NS	275.2±34.4***
$BaCl_2$	142.1±6.4**	154.0±14.2*	110.4±8.4 NS	216.0±24.7***
$CoCl_2$	132.1±8.7*	171.1±3.9***	91.9±0.05 NS	157.9±16.8*
$CdCl_2$	88.2±0.08 NS	135.7±8.8*	77.6±1.2 NS	274.7±109.6***
$MgCl_2$	110.5±8.9 NS	71.3±2.8*	71.7±2.6 NS	297.9±58.5***
$CuCl_2$	28.5±2.7***	31.9±2.3***	119.7±8.0 NS	233.1±40.8***

Results are expressed as percentage of ruminal enzyme activity, considering 100% activity obtained when ruminal fluid was incubated without divalent salts. Data are mean of six determinations ± S.E.M.
*P<0.05, **P<0.01, ***P<0.001 and NS not significant

The higher assayed concentration (20x10^{-3} mol/L) of Cu^{2+} and Zn^{2+} produced a high inhibitory effect (from 50 to 70 % of control ALT and AST activities). Salts of divalent ions such as Ba^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and Mg^{2+} produced a high activation effect (from 216 to 297 % of control GDH activity) when assayed at a concentration of 20 x 10^{-3} mol/L.

Negligible activation/inhibition of total ALT activity was detected when Cd^{2+} and Mg^{2+} were assayed. Enzyme GGT was the only enzyme tested that was insignificantly influenced by the assayed divalent ions.

DISCUSSION

The results showed that salts of divalent ions can affect the activities of several ruminal enzymes. Data from this study indicate that the effects of the assayed divalent ions depend on their relative concentration and the enzyme assayed. These substances had mostly stimulatory effect, especially when assayed at a relative concentration of 5 x 10^{-3} mol/L.

Ions, like copper, cadmium and zinc are well-known to interact with the sulphydryl group in the protein or enzyme, or they displace the metal ions from the enzyme, thereby interfering with enzymatic activity.

Forsberg (1978) observed that Cu concentrations of 21 μg/mL could *in vitro* decrease the fermentative activity and growth of certain populations of bacteria. Later Wallace and Mc Kain (1996) reported that copper, chromium and mercury inhibited *Prevotella ruminicola* dipeptidase activity to 15 and 5 % of control activity, respectively in *in vitro* experiment. Similar results were found by Fahmy *et al.* (1998) who described the effectiveness of heavy metals as inhibitors of the camel

rumen urease at the concentration of 5 x 10^{-3} mmol/L was, in decreasing order $Hg^{2+}>Cu^{2+}>Ni^{2+}>Co^{2+}$ with 97, 94, 61 and 7 % inhibition, respectively.

Results from our study indicate that copper was found to inhibit alanine aminotransferase and aspartate aminotransferase, when assayed at a higher concentration, and on the other hand, it appeared to stimulate gamma-glutamyltransferase, when asssayed at a lower concentration.

Results of further experiments showed that copper could have stimulatory effects on some enzyme activities (Zaki *et al.,* 2002; Faixová *et al.,* 2004), whereas Engle and Spears (2000) reported that the effect of copper on ruminal enzyme activity *in vivo* is negligible.

Results of our study showed that zinc showed an activation effect on total ALT, AST and GDH activities when assayed at a lower concentration and cobalt (except GGT activity) stimulated the activities of all enzymes tested at both concentrations. This is in agreement with findings of Wallace and Mc Kain (1996) who reported that cobalt, manganese and zinc stimulated *P. ruminicola* dipeptidase activity by 189, 30 and 26%, respectively. On the other hand, Fahmy *et al*. (1998) have observed that zinc, when assayed at a relative concentration of 5x10^{-3} mol/L, inhibited the camel rumen urease activity by 90%. These results are also consistent with findings of Martinez *et al.* (2002) who reported that salts of divalent ions such as Sn^{2+}, Hg^{2+}, Ni^{2+} and Zn^{2+} produced 90, 82, 65 and 44 % inhibition of amylase activity, respectively when assayed at a relative concentration of 5 x 10^{-3} mol/L. Zinc is the integral component of about 200 metalloenzymes, including carbonic anhydrase, alcohol dehydrogenase and glutamate dehydrogenase.

Results of our study indicate that salts of divalent ions such as Cd^{2+} and Mg^{2+} could affect the activities of several ruminal enzymes. Cadmium produced activation effects on AST and magnesium had stimulatory effects on GDH activity when both concentrations were tested.

Thus, some metal ions are potent both inhibitors and activators of ruminal enzymes, their toxicity and other aspects of the fermentation proless with respect to the maximum dietary content remains to be determined.

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UTICAJ DVOVALENTNIH JONA NA AKTIVNOST ENZIMA U BURAGU OVCE

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SADRŽAJ

U ovom radu su prikazani rezultati ispitivanja uticaja hloridnih soli dvovalentnih jona na aktivnost sledećih enzima iz sadržaja buraga ovce: alanin aminotransferaze (ALT), aspartat aminotransferaze (AST), gama-glutamiltransferaze (GGT) i glutamat dehidrogenaze (GDH). Sadržaj buraga je dobijan od 6 ovaca kojima je prethodno urađena kanulacija buraga. Ispitivan je uticaj $ZnCl_2$, $CuCl_2$, $MgCl_2$, $CdCl_2$, $BaCl_2$ i $CoCl_2$ na aktivnost navedenih enzima posle inkubacije sadržaja buraga sa rastvorima ovih soli u trajanju od 30 min. na temperaturi od 37°C. Korišćene su dve koncentracije rastvora soli: 5 i 20 x 10^{-3} mol/L. Veća koncentracija soli bakra i cinka dovela je do smanjenja aktivnosti ALT i AST za 50-70%. Manja koncentracija $CuCl_2$ smanjila je aktivnost GDH za 57%. Soli dvovalentnih jona barijuma, bakra, kadmijuma, cinka i magnezijuma u koncentraciji od 20 x 10^{-3} mol/L ispoljavale su aktivatorski efekat prema GDH u opsegu od 216 – 297%. Niže koncentracije soli kobalta i cinka ispoljavale su aktivatorski efekat prema ALT a soli kadmijuma, cinka i kobalta su dovodile do aktivacije AST u opsegu od 127-172%. Ovi rezultati ukazuju da efekti ispitivanih hloridnih soli dvovalentnih jona na aktivnost enzima u buragu zavise od njihove relativne koncentracije i vrste enzima.