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Production of protected amino acids using the reaction between hydroxycarboxylic acids and amino acids as well as binding on the bentonite

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Abstract. We have developed methods for the production of protected methionine and protected lysine, making use of the reaction between citric acid and malic acid as well as methionine and lysine, on the one hand, and of the interaction between swollen bentonite and the two amino acids, on the other hand. Our in vivo and in vitro experiments have demonstrated that one part of the amino acids transformed during the reaction, while another part bound on the bentonite's surface to a significant degree. Assisted by the reaction between hydroxycarboxylic acids

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and amino acids, we achieved a protection of about 75% for methionine and 60% for lysine, that is, 25% of the methionine and 40% of the lysine appeared in the free amino acid fraction. The swollen bentonite bound 75% of the added methionine and 60% of the added lysine. Our chemical analyses have demonstrated that through the time–temperature combinations applied by us the methionine and lysine do not undergo significant degradation and can be fully released from the protected form. Further, our in vitro experiments using rumen fluid from fistulated cattle showed that during the average retention time of the fodder in the rumen the protected amino acids will resist microbial enzymes and maintain their protected status during their presence in the rumen.

1 Introduction

Our ruminant domestic animals can provide us with an adequate amount and quality of food products of animal origin if, on the one hand, we ensure for them degradable nitrogenous substances, first of all, protein, necessary for the development of the ruminal microorganisms, and, on the other hand, if we provide the animals with proteins and amino acids that do not degrade in the rumen but, at the same time, can optimally complement the amino acid composition of the proteins produced by bacteria. Once these two requirements are met, the ruminant animal (cattle) will be able to make maximum use of its genetic make-up to produce a great amount of good-quality milk and meat. Thus, rumen-degradable (RDP) and rumen-undegradable dietary protein (UDP) must achieve an optimal quantitative balance in ruminants' fodder (*Schwab*, 1995; *Rode* & *Kung*, 1996).

Proteins are the most valuable components of the fodder, wherefore their optimal utilization and their availability in the animal are indispensable. Optimal protein supply and proteins with appropriate amino acid composition make possible the production of a great amount of good-quality milk and meat protein.

A potential solution for this is the use of rumen-undegradable, protected proteins and amino acids (*Arambel et al.*, 1987; *Ayoade et al.*, 1982; *Buttery et al.*, 1977; *Kamalak et al.*, 2005; *Schwab*, 1995).

In dairy cattle, especially in the first trimester of lactation, protein is the limiting component in cattle fodder since the protein content of the dry matter in the fodder intake cannot satisfy the protein needs of dairy production (*Robinson et al.*, 1992; *Olmos Colmenero* & *Broderick*, 2006). The least efficient solution is fodder supplementation with more and more proteins as most

part of them is degraded by the ruminal microorganisms that build up their own proteins from the ingredients obtained this way. According to examinations (*Rode & Kung*, 1996), a mere 25–35% of dietary intake protein reaches the small intestine, wherefrom amino acids degraded by digestive enzymes may be absorbed. A much more effective way is to use so-called bypass proteins (rumen-undegradable intake protein, UIP), which ruminal microorganisms can not degrade, and so they can get to the small intestine (*Calsamiglia & Stern*, 1993). Yet another expedient solution may be the application of protected amino acids (rumen-protected amino acids, RPAA), which, among others, can counterpoise the ill-balanced amino acid composition of the fodder (*Chalupa*, 1975).

When it comes to animals, we cannot talk of protein needs but rather of essential and non-essential amino acid needs as these are the building blocks of their body proteins and contribute to producing foods of animal origin such as milk, meat, or eggs. In terms of dairy production, methionine and lysine enter the category of limiting amino acids as the protein produced by ruminal microorganisms cannot meet dairy production needs regarding these two amino acids, and thus it cannot satisfy the lysine and methionine needs of milk protein synthesis (*Broderick et al.*, 1974; *Lee et al.*, 2012, 2015).

This statement holds true for the amino acid content of microbial protein too, which is well-balanced for cattle needs and which covers approximately half of the amino acid needs of the cattle. Therefore, microbial protein is of fine quality for cattle, but in cases of high milk production lysine and methionine deficiency may develop (*Chalupa*, 1975; *Mepham*, 1982).

Protozoa's protein contains more lysine and less methionine compared to bacteria, but this difference is not significant as their contribution to meeting the amino acid needs of the cattle is not a substantial one (*Harrison et al.*, 1979). Microbial protein synthesis is limited by the energy necessary for fermentation and by the proportion of feed protein degradable in the rumen, which is why in many cases microbial protein cannot meet the amino acid requirements of animals with high milk production (*Doepel et al.*, 2004).

2 Literature review

Recently, a great many types of rumen-ungradable proteins have been applied in an effort to meet animals' amino acid needs. Through various technological treatments, these proteins were made more resistant to the microorganisms' enzymes (*Waltz & Stern*, 1989). However, the composition of these amino

acids leaves much to be desired as there are very few proteins with an optimal methionine and lysine content (*de Boer et al.*, 1978). First of all, heat treatment is applied to these protein resources, during which indigestible Maillard reaction products are created, while such cross-links are formed between the proteins that can resist the proteolytic enzyme of the microbes (*Ashes et al.*, 1984; *Broderick & Craig*, 1980). Nevertheless, heat treatment must be approached very cautiously as it may cause losses in the case of heat-sensitive amino acids such as lysine, methionine, or cystine.

Since there is no ingredient to be used for the production of microbial proteins, rumen-undegradable proteins can reduce the microorganisms' protein synthesis as well. Not any rumen-ungradable ingredient has been found that could fully satisfy the amino acid needs of dairy production; what is more, the majority of such proteins turned out to be deficient in several essential amino acids (*Wang et al.*, 2016). The best way to address this issue is to apply a combination of several proteins of this kind, which will complement one another. In addition, some experiments performed with rumen-undegradable proteins showed a reduced amount of milk and milk protein content; therefore, it is extremely difficult to assess the outcomes of feeding such protein (*Rossi et al.*, 2003).

Among essential amino acids, lysine is the limiting element when animals are fed grain-based fodder, whereas methionine becomes limiting when animals are fed leguminous plants or, formerly, fodder of animal origin. *Schwab et al.* (1992a,b) established that in terms of dairy production protected lysine is of greater importance at the culmination of lactation production when compared to protected methionine, while in mid-lactation period both of them are equally significant in this perspective. Perhaps, this can account for the differences found when cows in various lactation stages were fed exclusively protected methionine (*Vyas & Erdman*, 2009). *Schwab et al.* (1992a) observed that feeding with protected methionine and protected lysine increased dairy production and milk protein content, which was a more striking increase regarding casein, a rather essential element in terms of cheese production.

In some experiments, the amount of milk and milk protein content increased due to protected methionine and protected lysine, some other experiments found no changes in this respect (*Trinacty et al.*, 2009), while in most experiments amino acid supplementation did not influence milk fat matter (*Buttery et al.*, 1977). In a few cases, protected methionine increased fibre digestibility and the amount of milk fat; the methionine may have even played a role in milk fat synthesis and metabolism (*Giallongo et al.*, 2015). The aforementioned let us conclude that the effect of methionine depends on lactation conditions, fod-

der quality, and the animal's physical condition, wherefore we cannot clearly outline the actual effect (*Weber et al.*, 1992; *Donkin et al.*, 1989; *Patton*, 2010).

The amount of amino acid supplementation necessary for cattle is yet another factor that is hard to define sometimes. According to *Schwab* (1995), requirements can be expressed in absolute terms (g/day) or can be given in relative values, expressed as a percentage in comparison with the amino acid composition of the fodder. About lysine content, for instance, it is being maintained that it has to cover 15% of all absorbable essential amino acids, while for methionine this value is only 5.3%. These values are considerably higher than what is available for cattle under normal feeding conditions. Applying an indirect approach, *Socha* and *Schwab* (1995) found that lysine supplementation had a much greater impact on the amount of milk and milk protein content than methionine supplementation. When the amount of lysine was below 14% of the essential amino acids, methionine supplementation reduced both milk protein content and the amount of the produced milk protein. Therefore, assessing the rate of the optimal lysine and methionine supplementation for traditionally fed cows becomes particularly difficult (*Loerch* & *Oke*, 1989).

Free amino acids are quickly broken down by the enzymes of ruminal microorganisms, wherefore these are not suitable either for mitigating the amino acid deficiency of the cattle or for supplementing the protein having an inadequate amino acid composition. However, protected amino acids are not degraded in the rumen but produce their effect via absorption in the forward section of the small intestine. Several procedures have been worked out to produce protected amino acids, which need to meet the following criteria: resistance to microbial degradation, being released in the abomasum or the forward section of the small intestine, absorption in the small intestine, and contribution to meeting the animal's essential amino acid needs. Additionally, they should also withstand technological interventions and low silage pH and make their way safely to the small intestine.

Plenty of methods have been elaborated for the production of both the protected proteins and protected amino acids. The best known of these are heat treatment and chemical modification, but their combination is not a rare thing to come across either. An outstanding compilation of these methods can be found in *Schwab's* (1995) publication.

Studies on free amino acids quickly pointed out that in the growth stage of ruminants the two limiting amino acids of protein formation are lysine and methionine. Nitrogen retention experiments carried out on lambs found methionine to be the first and lysine the second limiting amino acid. Thus, in accordance with the foregoing findings, ruminants' primary needs are protected

lysine and protected methionine supplementation in order for their optimal growth and then milk production capacity can be made full use of (*Schwab* & *Broderick*, 2012).

A great number of procedures have been put together to produce protected amino acids. Most important of these are as follows: the application of amino acid analogues, or amino acid derivatives in feeding (*Amos et al.*, 1974; *Ayoade et al.*, 1982); lipid coating (*Neudoeeffer et al.*, 1971); encapsulation with pH-sensitive materials that withstand ruminal conditions but are decomposed in the abomasum and the small intestine, thus releasing the amino acids (*Schwab*, 1995).

In the early 1970s, the amino group or the carboxyl group was modified or such derivatives were created where the amino group was replaced by some other (e.g. hydroxyl) group, this way protecting the amino acid from rumen degradation (*Griel et al.*, 1968; *Jones et al.*, 1988). It is highly essential that such a protected derivative should transform in the small intestine into the original amino acid, be absorbed, and contribute to meeting the animal's amino acid needs (*Mir et al.*, 1984). Particularly in the case of methionine were such analogue compounds created and tested in the context of in vivo experiments. Most researchers investigated those derivatives where long-chain fatty acids were linked to the amino group, protecting it from rumen degradation (*Langar et al.*, 1978). Many studied the hydroxy analogue of methionine, the N-hydroxymethyl-DL-methionine-Ca and the di-hydroxymethyl-L-lysine-Ca. Since in the abomasum only a small amount of these derivatives reverted to the amino acid under discussion, they cannot be considered protected amino acids (*Weber et al.*, 1992; *Kenna* & *Schwab*, 1981).

Another possible method for developing the ruminal protection of amino acids is coating them with materials that can resist the microorganisms' enzymes and that are degraded in the abomasum alone or, further on, in the small intestine, during which amino acids are released from the coating. Fats and oils were frequently utilized to this end, often in combination with inorganic materials and carbohydrates, such as stabilizing agents, but plasticizers and fillers were also used to ensure protection. Since protection ensured their safe use, these materials were widely applied in practice, although many of them proved to be inadequate for the protection of methionine (*Loerch*, 1989).

The very first protected methionine was made up of 20% of DL-methionine, 20% of kaolin, and 60% of tristearin, of which 65% of the methionine was absorbed from the small intestine. From most similarly protected preparations, 70–80% of the methionine was absorbed and 20–30% of it was eliminated along with the faecal matter (*Lapierre et al.*, 2006, 2012). In the next step, such

coating materials were also developed on which amino acids are released in smaller quantities, making thus possible that an optimal amount of them be available at all times, bringing about a significant increase in their utilization.

Such pH-sensitive polymers have also been applied that maintain their stability in ruminal conditions but are degraded due to low abomasum pH, releasing the amino acids protected this way. Various polymers and copolymers were developed to suit this purpose, enabling the amino acids' protection against digestive enzymes during their presence in the rumen up to 48 hours.

In summary, it has become clear that the two limiting amino acids of meat and milk production are lysine and methionine, as the otherwise good-quality microbial protein does not contain a sufficient amount of any of them to satisfy the needs of animals with high milk production. This supplementation cannot be carried out with free amino acids as they are subjected to degradation by the microorganisms' enzymes, and microbial protein is re-created from degradation products. The only solution is the production and application of protected amino acids, which are able to withstand ruminal conditions prevailing in the rumen as well as microbial enzymes and which make their way to the abomasum and the small intestine to be released and become available for the animal to facilitate the production of large amounts of good-quality food of animal origin.

3 Justification and objectives of the experiment

The aim of our research was to create protected amino acids by applying a novel method, never used before and yet unknown to specialized literature. We tried to address the issue in two different ways. We attempted to produce protected amino acids via the chemical reaction between di- and trihydroxycarboxylic acids, on the one hand, and by exploiting the connections existing between clay minerals and amino acids, on the other. Our intention was to test amino acid 'protection' with in vitro and in vivo experiments.

4 Materials and methods

4.1 Experiments performed with citric acid and malic acid for the production of protected methionine and protected lysine

Dry heat treatment of amino acids and hydroxycarboxylic acids

1 g of citric acid was added to 9 g of lysine and the same amount of citric acid was added to 9 g of methionine, while in the subsequent experiment citric acid was replaced by malic acid. Heat treatment was performed at a temperature range of 140–170 °C for 30–60 minutes. Afterwards, the amount of citric acid and malic acid was increased to 50%, and heat treatment was repeated at 170 °C for 30–60 minutes.

Aqueous heat treatment of amino acids and hydroxycarboxylic acids

Following experiments performed under dry conditions, the mixture of citric acid, malic acid, and amino acids was admixed with an amount of distilled water sufficient to obtain a slurry, whereafter heat treatment was performed at a temperature range of 140–170 °C for 30–60 minutes followed by measuring the amount of free lysine and free methionine.

4.2 Production of protected methionine and protected lysine with the use of bentonite

Dry mixing of bentonite and amino acids

At first, we tried to link lysine and methionine to bentonite in a dry mixture for various durations. 20 g of lysine hydrochloride was mixed with 80 g of bentonite, ground, and then treated at 100 °C in an exsiccator for 5-10-15-20-30-40-50-60-120, and 180 minutes. The same experiment applying the same temperature and the same duration was repeated with methionine. Finally, the free methionine and free lysine content of the treated samples was determined.

Aqueous mixing of bentonite and amino acids

In the next series of experiments, bentonite was activated via swelling and suspension by distilled water so that the active groups can bind the amino acids under discussion. In the first stage of the wet experiment, the bentonite was swollen in distilled water overnight, and then a concentrated solution of methionine and lysine was added to the swollen bentonite. Thereafter, the mixture of bentonite and amino acids was suspended in an amount of distilled water sufficient to obtain a low-viscosity mixture, the resulting suspension was left to stand overnight, and the next morning it was subjected to heat treatment at 100, 150, and 200 °C for 5-10-15-20-30-40-50-60-120, and 180 minutes. In the course of heat treatment, particular attention had to be paid to choosing the moment of measurement carefully: the measuring process had to

be initiated once the water had been completely vaporized as the temperature of the wet sample can hardly be over 100 °C. Then, an analysis was carried out on the composition of the bentonite–amino acid complexes heat treated at various temperatures and created via wet procedure. Parallel to the distilled swelling experiments, we attempted to swell the bentonite–amino acid mixture in 0.1 mol as well as 1 mol hydrochloric acid, whereafter an analysis of amino acids was performed.

During our subsequent experiments, we added 40 g of lysine-hydrochloride and 300 cm^3 of water to 160 g of bentonite, and then we repeated the experiment with the same amount of methionine instead of the lysine-hydrochloride. With both amino acids, we obtained a slurry-like liquid mixture. We waited until the liquid had evaporated, and then the remainder was heat treated at 200 °C in an exsiccator for one hour. The obtained samples were utilized in two types of experiments.

4.3 In vitro experiment with living ruminal flora and fauna

1 g of lysine was added to 20 cm^3 of rumen fluid, containing living microorganisms, of a Holstein-Friesian cow found in its mid-lactation period and having a ruminal fistula. In a further experiment, 1 g of methionine was added to the same amount of rumen fluid, which we considered as a control sample. Following this, we repeated the above two experiments, this time using 'protected' methionine and lysine of the same amount as previously specified. The samples were incubated at 38–39 °C in an exsiccator for 16 hours, whereafter they were hermetically sealed and stored in a freezer until determination of the amino acids.

During the analysis of the rumen fluid samples, we added 2.5 cm^3 of 6% sulphosalicylic acid to 5 cm^3 of rumen fluid for the purpose of protein precipitation. After shaking it up and leaving it to stand for 10 minutes, we centrifuged it for ten minutes at 6,000 rotation/minute. Following centrifugation, we set pH to 2.2, but there was actually no need for us to set the pH because the pH of the ruminal fluid decreased exactly to this value upon addition of the sulphosalicylic acid. After protein precipitation and centrifugation, the solution remained turbid; therefore, the samples were filtered prior to determination of amino acids, and then we applied decimal dilution to determine the free methionine and free lysine content of the rumen microflora.

4.4 In vivo experiments with fistulated cows

The in vitro experiment was also performed with fistulated cows under in vivo conditions. The in vivo experiments were carried out on four fistulated cattle, applying the nylon bag technique, during which we added 10 g of 'protected methionine' as well as 'protected' lysine to the nylon bag. Following a 16-hour-long storage in the rumen and upon drying the bags, we measured their free methionine and lysine content. Throughout the experiment, we made use of the same samples that were used with the ruminal fluid.

4.5 Determination of the samples' amino acid composition with ion-exchange column chromatography

Amino acid analyser type INGOS AAA400 was used to determine the free lysine and free methionine content of the samples. The ion-exchange column was a 35 × 0.37 cm OSTION Lg ANB, containing sulphonated polystyrene synthetic resin cross-linked with divinylbenzene. The pH and concentration of the applied sodium citrate buffers varied as follows: 1: pH 2.7, 0.2 M Na^+ 0–29 minutes; 2: pH 4.25, 0.5 M Na^+ 29–44 minutes; 3: pH 6.9, 1.12 M Na^+ 44–66 minutes; 4: 0.2 M NaOH 66–71 minutes, equilibration with buffer 1: 71–101 minutes. The temperature programme was as follows: 0–29 minutes: 50 °C, 29–44 minutes: 60 °C, 44–66 minutes: 74 °C, 66–74 minutes: 60 °C, and 74–101 minutes : 50 °C. A detailed description of the method can be found in the work of *Csapó et al.* (2008).

5 Results and conclusions

5.1 Experiments performed with citric acid and malic acid for the production of protected methionine and protected lysine

Malic acid is such a hydroxydicarboxylic acid that may be capable of reactions with amino acids via both its carboxyl groups and hydroxyl group. Citric acid is a hydroxytricarboxylic acid with three carboxyl groups and one hydroxyl group, which may likewise be suitable for reactions with both lysine and methionine. Ester linkages might also be formed between the carboxyl group of the amino acid and the hydroxyl group of the hydroxy acids, but acid anhydride bond is also possible to take place between the carboxyl group of the amino acids and the malic acid as well as the carboxyl groups of the citric acids. In addition to the above, we may also consider a reaction of the

two and three carboxyl groups of the malic acid and citric acid, respectively, with lysine and methionine. Furthermore, we cannot exclude either that the carboxyl group of the hydroxy acids will form a quasi-peptide bond with the a-amino group of the amino acids or that one of the carboxyl groups of the carboxylic acids will react with the carboxyl group of the amino acid and the other one with the amino group of the amino acid, creating condensation polymers of higher molecular weight. In the case of lysine, a new possibility arises due to the e-amino group, which can also react with the carboxyl group of the carboxylic acids.

Dry mixing of amino acids and hydroxycarboxylic acids

Since the melting point of the malic acid is between 129 and 133 °C and that of the citric acid is 153 °C, we considered as a first step to melt a proper proportion of the mixture of the two amino acids and the two carboxylic acids at a temperature of 160–170 °C in the hope that a reaction would take place between the carboxylic acids and the amino acids. After a half-an-hour-long heat treatment at 140–170 °C, no reactions were observed whatsoever with either any of the amino acids or any of the carboxylic acids, meaning that the free amino acids were assayed by weighing with the help of amino acid analysis, in the same amount as the sample that was not subjected to heat treatment. Thereafter, the amount of citric acid and malic acid was increased to 50% and heat treatment was repeated at 170 °C for half an hour. Again, the dry heat treatment yielded no results, leading us to the conclusion that in solid phase, at 170 °C no significant reaction took place between the carboxylic acids and the amino acids despite that both carboxylic acids had melted.

Aqueous mixing of amino acids and hydroxycarboxylic acids

Following experiments performed under dry conditions, the mixture of citric acid, malic acid, and amino acids was admixed with an amount of distilled water sufficient to obtain a slurry, whereafter heat treatment was performed at a temperature of 170 °C for one hour followed by measuring the amount of free lysine and free methionine.

We analysed the following: the lysine content of the mixture dry treated (170 °C, one hour) with citric acid and malic acid; the methionine and lysine content of the samples treated (170 °C, one hour) with citric acid and malic acid, suspended in water; the composition of the samples treated with citric acid and malic acid; ran a full analysis of the free lysine content of the sample heat treated under aqueous conditions and of the lysine content after

hydrolysis by hydrochloric acid. Chromatograms reveal that in the case of the sample treated with citric acid there is no ninhydrin positive compound on the chromatogram other than lysine and that, apart from lysine, there is no degradation product present in a significant concentration even after hydrolysis by hydrochloric acid. The same treatments and analyses were performed with methionine as with lysine, while the amount of methionine also underwent analysis as to how it changed as a result of the various treatments.

During the experiment performed with methionine, increasing the concentration of the citric acid and malic acid was not particularly successful in the case of wet treatment because in both hydroxycarboxylic acids 75% of the methionine transformed into some kind of compound, it was not detectable from the free amino acid fraction, and 25% of it remained free in the case of both amino acids. The same was the case with lysine, except that approx. 60% of it transformed and 40% remained in the free amino acid fraction.

We performed 6M hydrochloric acid hydrolysis of the obtained compound at a temperature of 110 °C for 24 hours, and methionine as well as lysine concentration was measured following hydrolysis. Results looked promising as, subsequent upon hydrolysis, we have retrieved nearly 100% of the amino acids in question as compared to the control, meaning that amino acids did not break down during the reactions performed with carboxylic acids but merely transformed into such a product that could be transformed back into the amino acid under discussion by the hydrochloric acid hydrolysis applied in the process of determining the amino acid composition of the protein.

5.2 Production of protected methionine and protected lysine with the use of bentonite

In our experiments, amino acid analyser was applied to determine the lysine and methionine content of the utilized 'lysine-hydrochloride' as well as 'methionine'. The lysine content of the 'lysine-hydrochloride' was measured to be 79.48%, whereas the methionine content of the 'methionine' was 99.77%. Upon adding 20 g of lysine-hydrochloride to 80 g of bentonite, we obtained a lysine content of 15.91%, while upon adding 20 g of methionine to 80 g of bentonite resulted a methionine content of 19.97%.

Following a heat treatment performed at 200 °C for one hour, we could assay by weighing 9.73% of the lysine content of approx. 16%. Therefore, it appears that 40% of the lysine was bound by the bentonite under these experimental conditions, while 60% of it was still present in its free form in the sample. In the case of methionine, we could assay by weighing 4.93 g of the methionine

content of 20%, which means that nearly 75% of the sample was bound to the bentonite and only 25% of it remained in its free form.

After heat treatment at 200 °C, a full-scale analysis was performed with both the lysine and the methionine, during which we were interested in knowing whether the amount of amino acids would decrease while under heat treatment and whether any kind of by-product would be produced. In the course of the analysis, we established that lysine content remained practically unchanged throughout the heat treatment, while in the case of methionine we could detect in the sample a minimum amount of methionine sulphone, the oxidation derivative of methionine, which, however, did not reach 5% in any of the cases.

As we observed a protection of 40–80% in the case of both lysine and methionine, we performed the experiments with the use of ruminal fluid as well, where in the case of methionine and upon adding the control (non-protected) methionine, we could assay by weighing 0.87 g of methionine, whereas upon addition of protected methionine to the ruminal fluid the assay by weighing yielded 0.30 g. This means that approx. 65% of the methionine remained in protected form during the experiment with the ruminal fluid, that is, nearly 80% of the protected methionine preserved its protected form following the experiment with the ruminal fluid. This is due to the fact that only 75% of the material used was protected, 25% of it was in free form, which probably dissolved right away into the ruminal fluid, while in what followed a further 10% would dissolve from the 75% protected quantity, whereupon we assessed the protected status of the obtained material to be at 65% after exiting the rumen.

In the case of lysine, upon treatment with ruminal fluid, the lysine content of the control sample (pure synthetic lysine) was assayed by weighing 0.44 g, while the lysine content of the protected lysine was 0.35 g, which means that a total of 21% of the sample's lysine content remained protected after the treatment with ruminal fluid; thus, 80% of it dissolved into the ruminal fluid.

In terms of protection, the in vivo experiment yielded results that were 50% worse as compared to the in vitro experiment performed with ruminal fluid, an outcome most probably due to the fact that part of the methionine and lysine linked through the pores of the nylon bag to the fine, powdery bentonite fell out of the nylon bags mechanically, which reduced the amounts of protected methionine as well as protected lysine by 50%.

In conclusion, if the bentonite undergoes a minimum of 8-hour-long swelling process in (distilled) water, then, according to chemical analyses, it will become capable of binding 40% of the lysine and about 80% of the methionine

following heat treatment and upon 20% of added lysine and the same amount of added methionine. From this amount of lysine, 25% and from the methionine 65% remain bound to the bentonite even after treatment with ruminal fluid containing live rumen bacteria. When the experiment was performed with fistulated cows, protection decreased by 50%, which was first of all not owing to the different physiological conditions but rather to the fine, granular, protected, and bentonite-bound amino acids falling out of the bags.

6 Discussion of the results

An important question is whether the 40% of protected lysine and the 80% of protected methionine are sufficient amounts under conditions applied as previously described. We could possibly enhance the amino-acid-binding effect of the bentonite by increasing the concentration of the amino acid solution as well as the applied temperature. Heat should not be a concern as the examinations carried out have demonstrated that none of the amino acids undergoes relevant changes at 200 °C.

We could carry on the experiments with the rest of the essential amino acids. Leucine, isoleucine, and valine are highly resistant to all external effects, but certain problems may arise with threonine and tryptophan as both of them, especially the latter one, are extremely sensitive to acidic conditions.

Besides in vitro experiments, others could be performed too with the already protected amino acids and with the use of duodenal-fistulated cows, as all we know about these amino acids is that they are protected in the rumen, but it would also be an outstanding achievement if we could obtain some evidence of their absorption and availability in the small intestine.

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