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Degradation of two soluble proteins - casein and egg protein by a macro-*in vitro* method

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SUMMARY

Degradation of casein and egg protein were studied with whole rumen contents (RC) in a macro-*in* vitro system to elucidate previous findings of initial rapid disappearance of soluble proteins *in vitro*. Five to 7.5 kg of rumen contents from a dry and/or a lactating cow were incubated with buffer and casein or egg protein for 180 min with frequent sampling. Degradation was measured as loss of trichloroacetic acid precipitable N (TCA-N) from the inocula. Normal (39°C) and low (2°C) temperature incubations were examined in Exp. 1, using 1 g of TCA-N from casein. Four levels of casein (0-12 g TCA-N) in Exp. 2 and four levels of egg albumin (0-24 g TCA-N) in Exp. 3, were fermented at 39°C. Initial recovery of casein TCA-N was 106% at 2°C and 56% at 39°C (Exp. 1). Casein (TCA-N) recovered initially increased in Exp. 2 from 21% at 3 g to 86% at 12 g TCA-N, while absolute loss remained relatively constant at 358 mg TCA-N/kg RC (SD = 47). Fractional degradation rate was highest (0.03/min) at the intermediate dosage level. In the absence of rumen fluid (Exp. 4), no casein was lost. Initial egg protein recovery was on average 103% (Exp. 3). Recovery

seemed unaffected by dosage level, absolute degradation rate was relatively constant over time and increased with dosage level (P<0.001) from 1.48 to 2.95 mg TCA-N/(kg RC/ x min). Maximum degradation rate (mg TCA-N/(kg RC x min)~~TCA-N/kg RC/min~~) and affinity constant (mg TCA-N/kg RC-)were estimated at 261 and 1650 , respectively. It is concluded that a surprisingly constant amount of casein disappears immediately from warm rumen fluid and that this does not occur either with chilled rumen contents, in the absence of rumen fluid or when replaced with egg protein. The mechanisms for this disappearance are yet to be discovered.

Running head: Soluble protein degradation *in vitro*

Keywords: Macro in vitro; Protein degradation; Rumen; Casein; Egg albumin

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Introduction

Protein quality is an important variable in optimization of ruminant diets. Even though lack of key amino acids may limit milk production, the proportion of feed protein degraded in the rumen (effective protein degradation) and the availability of the feed protein passing from the rumen are normally the major protein quality criteria in use. Degradability of protein can be estimated *in vivo*, but these techniques are expensive, time-consuming and labour intensive.

There are also considerable problems inherent to these methods in separating feed and microbial protein and in evaluating proteins from single feeds.

In most evaluation systems, the soluble fraction is considered to be completely degraded in the rumen (*e.g.* Sniffen et al., 1992). Findings by Volden et al. (1998) showed, however, that approximately 10% of infused free amino acids may escape the rumen. A soluble protein such as casein with an assumed fermentation rate of 1/h and a liquid passage rate of 0.16-/h would result in a rumen escape of approximately 15%, whereas with the slower fermentation rate of soluble rapeseed cake protein (0.19 /h), the estimated escape would be 46% (Hedqvist and Udén, 2006).

The method of Hedqvist and Udén (2006) is a relatively simple method, similar to that of Broderick (1987) but without the use of bacterial growth inhibitors, to measure soluble protein degradation. Remaining soluble protein is precipitated from the bacteria-free supernatant to recover remaining feed proteins after fermentation. One major problem was encountered in this study. The recoveries of soluble proteins from 11 sources at 20 or 30 min were low and on the order of 25 to 36% and after 1 to 2 h of incubation, levels became difficult to separate from background.

A novel macro-*in vitro* method was introduced by Udén (2011) in a study on volatile fatty acid production. In this system, 5 to 10 kg of whole rumen contents can be used which enables continuous sampling of fluids and ensures a more rumen-like environment as compared to regular micro-systems using small amounts of strained rumen fluid.

The aim of this study was therefore to explore the macro-*in vitro* system for studying degradation of two soluble proteins and to investigate the phenomenon of initial disappearance of proteins from solution.

Materials and methods

Three *in vitro* rumen experiments and one experiment without rumen fluid were made to study the degradation of soluble proteins. Exp. 1 was a pilot study to confirm previous findings of low recoveries of soluble proteins both *in vitro* and *in vivo* (Hedqvist and Udén, 2006) and to study the effect of normal and low temperature on initial disappearance. In Exp. 2, four levels of casein and in Exp. 3, four levels of egg albumin were studied at normal rumen temperature. To test the possibility of attachment of casein to feed particles, casein was incubated with silage at normal rumen temperature in Exp. 4.

Proteins and inocula source

The casein used was bovine sodium caseinate (Sigma, CAS 9005-46-3; Sigma-Aldrich Sweden AB, Stockholm, Sweden) and the egg albumin was 'Egg Protein 80' (Olimp Sport Nutrition AB, Partille, Sweden). One dry and one lactating cow, fitted with rumen cannulas, were used as donors of rumen contents (RC). The dry cow consumed daily 3.4 kg dry matter (DM) grass hay with a crude protein content (CP) of 155 g/kg DM and 1.6 kg concentrates (CP: 200 g/kg DM). The lactating cow consumed 15 to 16 kg DM from a grass dominated silage (CP: 161 g/kg DM) and 7 kg DM of a concentrate mix (CP: 200 g/kg DM). All cows had free access to water and salt licks.

The *in vitro* system

A macro *in vitro* system was used for all incubations as described by Udén (2011), but with the following modifications: i) eight instead of two tubes with the inner dimensions (cm) 76(height) x 18.8(diameter) made of 6 mm hard polyethylene, ii) the insulated heated box was

made from 5-cm Styrofoam™ with the inner dimensions (cm) 110(length) x 55(width) x 60(height), iii) the mixer consisted of a grain auger (cm) 50(length) and 10(diameter) with 8 turns/m and a 45-cm shaft extending through the centre hole of the lid, and iv) the whole assembly including heater was mounted on a pallet for easy transport between *in vitro* room and stable.

The following general protocol was used for the *in vitro* incubations:

1. Day before the experiment: prepare sufficient quantities of McDougall's buffer (McDougall, 1948). Fill glass jars with 2-L of the buffer (blank) or the protein in question dissolved in the same amount of buffer. Record tare weights of *in vitro* tubes and assemble the *in vitro* unit. Add 2 L of pure McDougall's buffer to all tubes, gas with CO_2, seal the lids, and heat over night with the thermostat set at 39°C. Bubble CO_2 in all solutions over night.
2. Experimental day: add more CO_2 and move *in vitro* assembly to the stable and plug in heater. Record rumen pH and transfer whole RC from rumen fistulated cow(s) to each *in vitro* tube with minimum exposure to air.
3. Move assembly back to the *in vitro* room and plug in heater again. Add more CO_2 and pour the first solution (blank or protein solution) into *in vitro* tube no 1.
4. Start timer, mix the *in vitro* contents vigorously for 30 sec and take a 50-mL sample of the liquid fraction by aspiration (see Udén, 2011). Record pH and cool sample rapidly in ice bath. Continue with the tube no 2 and solution no 2, etc. Space samplings 2.5 min apart, which will allow for a minimum time interval of 20 min for each tube when using a total of 8 tubes.
5. Sample again according to schedule and continue sampling for a maximum of 3 h.
6. Centrifuge samples at 1500 x g for 5 min and split the supernatant into one 30- and one 15-mL sample. Transfer supernatants to refrigerator (4°C) and analyze for total N, NH_3-N and

118 trichloroacetic acid precipitable N (TCA-N) following morning as specified under 'Sample
119 analyses'.
120 7. Record weights of the *in vitro* tubes and determine DM concentrations of remaining
121 ingesta.
122
123 Experiment 1
124 Two separate incubations with two tubes each were made. Rumen contents from the lactating
125 cow was removed and the amount adjusted to 5 kg (Table 1) for each *in vitro* tube in both
126 incubations. In both incubations, 7.25 g dissolved casein (air dry weight) was added to one of
127 the tubes and only buffer to the other (blank). In the first incubation, the protocol described
128 above was followed, except that the amount of buffer used in Step 3 was only 1 L. Samples
129 were taken at 3, 30, 60, 90, 120 and 180 min during the incubation. In the second incubation,
130 the procedure differed on the following accounts. Rumen contents, buffer, casein solution and
131 *in vitro* assembly were chilled to 2°C before adding the casein or blank solutions to the RC.
132 These tubes were then kept in the insulated box with ice bags to maintain the 2°C temperature
133 during the entire measurement period. Samples were taken at 3, 60, 120 and 180 min.
134
135 Experiment 2 and 3
136 Casein was incubated at 0, 20, 40 or 80 g/tube in duplicate (a total of 8 tubes) in Exp. 2 and
137 egg powder was used in Exp. 3 at the levels of 1, 60, 120 and 240 g/tube (Table 1). The
138 higher levels used for egg protein were due to a suspicion that detection of any saturation
139 kinetics would require higher levels of this slow-degrading protein as opposed to casein.
140 Incubation times used were 2, 20, 40, 60, 80, 120 and 180 min in Exp. 2 and 1, 18.5, 38.5,
141 58.5, 91, 121 and 181 min in Exp. 3. Approximately 7 kg RC from the dry cow was used for

one of the replicates and RC from the lactating cow was used for the other. The amounts actually incubated are shown in Table 1 and samples were handled as in the protocol above.

Experiment 4

One-hundred grams of chopped silage with a dry matter content of 346 g/kg were placed in three Erlenmeyer flasks and heated in an oven at 60°C oven overnight to eliminate bacterial activity. On the following day, flasks were equilibrated to 39°C in a water bath, followed by the addition of 600 mL sterile 39°C McDougall's buffer containing either (two flasks) 1000 mg casein or no casein (one flask; blank). Sub-samples (35 mL) were taken after 3, 30, 90 and 180 min and handled as described above.

Sample analysis

In vitro tube RCs remaining after the incubations were dried at 55°C and weighed to estimate liquid volume in each tube. Total liquid volumes were calculated after adjustment for volumes removed during sampling.

In vitro supernatants were analyzed for NH_3-N in Exp. 2 and 3 (only for the 0, 60, 120 and 180 min samples), and in all samples for total soluble N and for TCA-N (except for Exp. 4). Liquid samples from the previous day (Exp. 1-3) were first centrifuged at 25000 x *g* for 15 min at 4°C. From the 15-mL supernatant, 3 mL ~~was~~ were taken for NH_3-N analysis and 7 mL w~~as~~ere frozen and saved. Ammonia-N was analyzed by the phenol-hypochlorite assay using flow-injection analysis (FIAstar™ 5012, Foss Analytical, Hillerød, Denmark).

The 30-mL sample was split into 12.5 and 10 mL sub-samples. To the 12.5-mL sample, 1.5 mL TCA (1000 g/L) was added and the mixture was put on ice for 1 h. This was followed by centrifugation at 25000 x g for 15 min at 4°C. Both the supernatant after precipitation with

166 TCA and the untreated supernatant (10 mL) were transferred to glass test tubes for N analysis
167 by the Kjeldahl procedure (Foss Tecator 2020 Digester and 2400 Kjeltec Analyzer unit,
168 Hillerød, Denmark), using copper as a catalyst. Amounts of TCA-precipitable N (TCA-N)
169 were calculated by difference.

170

171 Biometric analysis

172 All NH_3- and TCA-N values were blank corrected after adjustments for volume differences
173 among the *in vitro* blank and treatment tubes. Soluble N values were only recorded in Exp. 4
174 and were blank corrected before calculating recoveries.

175 As exponential degradation was assumed ~~used~~ for the disappearance of casein TCA-N (Exp.
176 2), a single exponential function was fitted to the individual data from all 6 treatment tubes by
177 TableCurve^{TM}TD (Jandel Scientific, San Rafael, CA, USA), using standard least-squares
178 minimization:

179 $Y = a + bxe^{(-c*t)}$ where,

180 'Y' = recovery of TCA-N, 'a' = curve asymptote, 'b' = y-axis intercept, 'c' = degradation rate
181 (/min) and 't' = time (min).

182 Both egg protein disappearance (Exp. 3) and NH_3-N appearance (Exp. 2 and 3) were linear in
183 fashion during the 3-h measurement periods and did not fit the exponential equation that was
184 used for casein results. Therefore, only linear regressions were fitted to the 6 treatment data
185 sets using Minitab® v. 15 (Minitab Inc., State College, PA, USA) and tested for similarities
186 among slopes.

187 To test if saturation kinetics prevailed, the Michaelis-Menten equation was fitted to both
188 protein sources, combining data from the two cows and three protein levels. Degradation
189 velocity for egg protein was calculated as the slope of the TCA-N concentration (mg N/kg

RC) *versus* dosage (mg TCA-N/kg RC; n=6). Casein degradation was calculated as the change in TCA-N concentration (mg N/kg RC) between each measurement *versus* remaining casein concentration (mg TCA-N/kg RC; n=42) at the beginning of each sampling period. Maximum degradation rate (V_{max}; mg TCA-N/(kg RC x min)~~TCA-N/kg RC/min~~) and the affinity constant (k_m; mg TCA-N/kg RC) were estimated using the Solver function in Microsoft Excel® to minimize Σ(estimated – measured rate)^2.

Data of degradation rate as affected by initial protein level was analyzed by simple regression analysis using Minitab®. Significant polynomial effects were considered at probabilities less than 0.05.

Results

The casein contained 148 mg and the egg powder 103 mg TCA-N/g air dry matter. Data on achieved dosage levels, weights of RC collected and total fluid volumes in the *in vitro* vessels are shown in Table 1.

Experiment 1

The recovery of a low dosage of casein TCA-N at two temperatures is shown in Fig. 1. There was a marked difference in the recovery at 2 and 39°C at the first sampling time (3 min) with a value of 106% at 2°C and 56% at 39°C. Corresponding recoveries at 30 min were 88 and 14%, respectively.

Experiment 2 and 3

Initial recovery of TCA-N

Recoveries of casein TCA-N at the first sampling time (2 min) increased dramatically with increasing dosage from 16 and 27% at approximately 500 mg/kg RC (Treatment 3) to 84 and 87% at approximately 1700 mg TCA-N/kg RC (Treatment 12) with RC from the dry and lactating cow, respectively (Fig. 2). In absolute terms, this recovery equaled a mean loss of 358 TCA-N mg/kg fresh weight RC after one minute of incubation and was relatively constant for all dosage levels (SD = 47; n = 6). Egg protein TCA-N recovery after 1 min was on average 103% and seemed unaffected by dosage level (Fig. 2).

Degradation of TCA-N

Curves for net TCA-N are shown in Fig. 3 and 4. Casein disappeared in a curvilinear fashion and fractional disappearance rates ('c') changed with dosage level. Rate *vs*. dose level was explained by a polynomial regression (R^2=0.977) where the quadratic term of the regression equation differed from zero (P=0.002).

Egg protein degradation was relatively constant over time for both inocula. Degradation increased linearly with dosage level (linear term: P<0.001) from an average of 1.48 for the lowest level to 2.95 mg TCA-N/(kg RC x min) ~~TCA-N/kg RC/min~~ for the highest dosage level (Table 3). The increase appeared to be curvilinearly related to dosage level, suggesting saturation kinetics. Applying the Michaelis-Menten equation to the egg protein data, resulted in a V_{max} estimate of 261mg TCA-N/(kg RC x min)~~TCA-N/kg RC/min~~ and a k_m of 1650 mg TCA-N/kg RC as shown in Fig. 5. The casein data did not fit the Michaelis-Menten equation. Initial rates differed markedly from subsequent rates and even after removal of all first observations, disappearance was directly proportional to vessel concentrations.

Ammonia production

Ammonia-N evolutions are also presented in Fig. 3 and 4 and showed correspondence with the differences in TCA-N disappearance of the two proteins. The increases in concentrations

over time were similar for all dosage levels and approximately linear for both proteins, with the exception for the lowest casein dose (Treatment 3), which seemed to level off after 60 min. Removing casein values for Treatment 3 at time > 60 min and regressing ammonia levels against fermentation time, gave slopes of 1.44±0.061 and 0.52±0.023 mg NH_3-N/(kg RC/ x min) (R^2 > 0.95) for casein and egg protein, respectively.

Experiment 4

The incubation of casein without rumen fluid showed that recovery of soluble N did not change over time and was on average 99% with a range of 95 to 104%.

Discussion

Measuring protein degradation has been a great challenge over the past decades and no method has yet received wide acceptance by the scientific community. The *in sacco* technique (e.g. Mehrez and Ørskov, 1977) is the dominating method for estimating rumen protein degradability. The method is labour intensive, cannot be applied to soluble proteins and has inherent problems with microbial N contamination and feed particle loss of undegraded N from the bags (Lopez, 2005).

Various *in* vitro systems have been introduced over the past decades. Some of their major shortcomings are listed in Table 4. In the Cornell Net Carbohydrate and Protein System, Sniffen et al. (1992) separated crude protein into five fractions based on their buffer and detergent solubility and linked these with estimated *in vitro* enzyme degradation rates (Krishnamoorthy et al.,1983). The method is attractive for reasons of low cost and simplicity but doubt exists as to the similarity of fungal enzyme and bacterial protein degradation. The inhibitor *in vitro* (IIV) system of Broderick (1987) measures appearance of amino acids and

ammonia in the presence of inhibitors of bacterial protein synthesis. The method is suitable for incubations of approximately 3 to 4 h and can give sufficient data for meaningful rate estimates, provided that protein degradation rates are high. The gas *in vitro* method of Raab et al. (1983) estimates protein degradation from ammonia evolution at graded levels of carbohydrate additions but is both expensive, complex and time consuming in its original form. The authors have presented mainly 24-h incubations and short incubation times were not investigated to any degree. Some improvements to this method were made recently by Karlsson et al. (2009) and Lorenz et al. (2011) to estimate degradation continuously up to 30 h in the same flask. However, the method has not yet been developed as a reliable routine method for protein rate estimations.

Soluble proteins have generally been regarded as being instantly degraded in the rumen (Krishnamoorthy, 1983) even though Volden et al. (1998) demonstrated that even amino acids could escape rumen fermentation at a level of 10%. These proteins can be separated from bacterial protein by high-speed centrifugation and measured as loss of proteins from solution (Hedqvist and Udén, 2006) without the need of inhibitors of protein synthesis, which should be an advantage in terms of maintaining bacterial activity over a longer time compared to the IIV method.

In the present study, the normal size *in vitro* system of approximately 50 mL was abandoned in favor of a macro system with whole rumen contents. This was done in order to create an environment *in vitro* as close to *in vivo* conditions as possible. The major drawbacks are a limited fermentation time of approximately 3 h, only applicable to soluble proteins and a reduced number of vessels/run. The large proportions of soluble proteins disappearing before 30 min (1st sampling) of fermentation in the study of Hedqvist and Udén (2006) were also confirmed for casein in the present study. Disappearance occurred before 3 min of incubation but at 2°C, recovery was approximately complete (Fig. 1) and recovery was also

approximately 100% when no rumen fluid was present. Similar casein levels were used in Exp. 1 (approximately 250 mg N/L rumen fluid) as in the study of Hedqvist and Udén (2006) where levels ranged from 100 to 200 mg N/L strained rumen fluid. When casein levels were further increased in Exp. 2, initial recoveries improved asymptotically (Fig. 2) and from these data, a loss of approximately 358 mg TCA-N /kg fresh weight of RC was evident. Adsorption of soluble protein was reported to occur also at low temperatures when proteolytic activity is at a minimum (Nugent and Mangan, 1981; Wallace, 1985). Wallace (1985) reported a maximum binding capacity of 10 µg casein/mg bacterial protein. However, in the study of Hedqvist and Udén (2006), this level was estimated to account only for an initial binding of 6% and could not explain the large initial casein loss. Casein structure is unique and caseins are highly surface active forming both micelles and gels under certain conditions (Horne, 2002). The drastic reduction in initial disappearance when cooling the RC in the present study can presently only be explained by attachment. It is unlikely that regular precipitation would have been higher at 39 than at 2°C.

Protein degradation involves a number of steps before the protein has been completely metabolized. Casein *in vitro* data from Broderick and Craig (1989) was used by Udén (2000) to formulate a model consisting of three extracellular (protein, peptides and amino acids), one intracellular (amino acids) and one total ammonia N-pool. Parameter estimations of the model revealed that degradation of casein to peptides and the uptake of peptides had similar rates. It was therefore concluded that estimating ruminal protein degradation from the appearance of N in the form of fermentation end-products (ammonia and amino acids) may not be correct using a one-pool model.

As protein degradation is defined according to method employed, rate estimates may differ irrespective of proteolytic activity. Appearance of amino acids and/or ammonia is used in both the IIV system of Broderick (1987) and in the gas-*in vitro* system of Raab et al (1983).

Loss of TCA-precipitable N was used in the method of Hedqvist and Udén (2006) and in the present study. In electrophoretic studies, loss of specific proteins are defined as degradation (e.g. McNabb, 1994; Messman and Weiss, 1994). Using appearance of *e.g.* ammonia by the gas-*in vitro* method includes both the degradation of protein to peptides and amino acids, absorption and catabolism. Disappearance measurements by *in vitro* or electrophoretic methods assume that degradation has occurred when the protein has been sufficiently reduced in size to resist precipitation, or is too small for electrophoretic detection. As an important goal of protein degradation measurements is the prediction of feed amino acid delivery to the small intestine, it seems logical that residual feed N in all forms from amino acids to intact proteins should be the ideal target. All present *in vitro* methods based on protein loss, ignore degradation beyond the lowest size detection limit which is particularly serious for the electrophoretic methods.

Fractional disappearance of casein (TCA-N) was rapid (0.91 to 1.75 /h) in the present study and highest at the intermediate level (Table 2). The reason for a higher fractional degradation at the intermediate level is not easily understood. Levels used were approximately equivalent to 270 to 1020 g of soluble protein to a cow with 100 kg of rumen contents. Within this range of casein levels, no evidence could be found for any saturation phenomena. Broderick and Clayton (1992), however, found evidence of saturation kinetics for casein degradation with similar levels as in Exp. 2 using the inhibitor *in vitro* method. When they estimated rate as V_{max}/k_m from this experiment, casein degradation rate approached 1.0 /h. This rate estimate should be the theoretical rate at an infinitesimal substrate level. Conventional rate estimations have shown considerably lower rates, normally less than 0.4 /h (Broderick and Clayton, 1992; Broderick et al., 2004a, b).

Both inoculas displayed similar degradation rates. The lactating cow consumed 4.5 times (22 to 23 kg DM) as much feed as the cow at maintenance but, as the comparison was restricted to

two cows, it cannot be regarded as a final proof of no difference. In the study of Broderick et al. (2004a) using casein, solvent soybean meal and expeller soybean meal, two feeding levels (1X and 3X maintenance) and three inocula sampling times (0, 2 and 4 h after feeding), there was an overall effect of feeding level ($P<0.001$) as well as a time and a time x level effect of the inocula on all individual feeds ($0.001<P<0.032$).

Egg protein initial recoveries seemed unaffected by levels above 800 mg N/kg RC, but the choice of protein levels prohibited detection of any minor protein losses. Ovalbumin is found mainly in egg white and its structure belongs to the serpin family. The proteins in this family are known for binding to enzymes and immobilizing them, but ovalbumin itself does not possess any protease inhibitory effects (Huntington and Stein, 2001). In spite of this, degradation of ovalbumin is very slow (McNabb et al., 1994) which was confirmed in the present study. McNabb et al. (1994) found a discrete lag phase of 16 h and a rate of 0.08 /h for the intact protein bands in their *in vitro* study. In the present study, no obvious lag phase was seen and degradation proceeded linearly within the 3-h incubation. The fact that egg protein rate seemed to display saturation kinetics (Fig. 5) in contrast to casein (Fig. 6) may suggest limited microbial enzyme capacity for this protein.

Conclusions

Casein behaved totally different from egg protein by displaying an immediate disappearance from the precipitable fraction of the *in vitro* supernatant at a level of 358 TCA-N mg/kg fresh weight of RC. For egg protein and for casein incubated at 2°C, initial recovery was nearly complete. Increasing casein dosages made estimates of recovery more reliable, but the estimated rate differed with dosage and was highest at the intermediate level (6 g N/tube). Egg protein degraded linearly and displayed evidence of saturation kinetics, whereas this could not be demonstrated for casein. The macro-*in vitro* system appears to be a promising in

terms of ease of handling and operates under more rumen-like conditions. The drawbacks are reduced number of vessels and that only short incubation times with soluble substrates are presently possible

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Table 1 Dosage levels of casein and egg protein, amounts of fresh rumen contents (RC) and liquid volumes obtained from a dry and/or lactating cow in Exp. 1 to 3. Treatment numbers refer to approximate TCA-N dosages in g/tube

	Dosage			Dry cow			Lactating cow		
Treatment	Air dry weight g	Soluble N g	TCA-N g	RC kg	Liquids* L	TCA-N† mg/kg RC	RC kg	Liquids* L	TCA-N† mg/kg RC
Exp. 1 - casein									
0	0	0	0				5.00	7.21	0
1	7.25	1.09	1.07				5.00	7.28	214
Exp. 2 - casein									
0	0	0	0	6.80	10.05	0	6.53	9.67	0
3	20	3.02	2.96	6.92	10.19	428	6.35	9.56	466
6	40	6.04	5.92	6.94	10.22	853	6.51	9.75	909
12	80	12.08	11.84	7.31	10.47	1620	6.46	9.65	1830
Exp. 3 - egg protein									
0	0	0	0	7.65	10.79	0	7.48	10.59	0
6	60	7.14	6.42	7.75	10.60	828	7.34	10.40	875
12	120	14.28	12.84	7.66	10.62	1680	7.46	10.46	1720
24	240	28.56	25.68	7.01	9.90	3660	7.56	10.40	3400

*Total liquid volumes after addition of 3 (Exp. 1) or 4 (Exp. 2 and 3) litres of McDougall's buffer

†Trichloroacetic acid

Table 2 Exp2: Parameters obtained from fitting an exponential function* to the recovery data of casein TCA-N (Fig. 3) from *in vitro* incubation with rumen contents from a dry and a lactating cow and three levels of casein (3, 6 and 12 g of TCA-N/tube)

	Dry cow			Lactating cow			P^B=		R^2
Treatment level:	3	6	12	3	6	12	L	Q	
a	-0.029	0.013	0.022	0.017	0.024	0.048			
b	0.211	0.614	0.817	0.292	0.582	0.829			
c (/min)	0.0212	0.0292	0.0152	0.0205	0.0276	0.0172	0.269	0.002	0.977
c (/h)	1.27	1.75	0.91	1.23	1.66	1.03			
R^2	0.940	0.990	0.998	0.997	0.996	0.999			

*$Y = a + bxe^{(c*time)}$; ^{B}P = probability for linear (L) and quadratic (Q) treatment effects.

Table 3 Exp 3: Linear regression of TCA-N (mg/kg rumen contents) upon time (min) from *in vitro* incubation of egg protein (Fig. 4) with rumen contents from a dry and a lactating cow and three levels of egg protein (6, 12 and 24 g of TCA-N/tube)

	Dry cow			Lactating cow			P*=	R^2
Treatment:	6	12	24	6	12	24	Linear	
Intercept	732	1556	3569	829	1679	3418		
Slope	-1.59	-2.19	-2.89	-1.37	-2.03	-3.01	<0.001	0.969
R^2	0.89	0.88	0.88	0.97	0.94	0.68		

*P = probability for linear treatment effects.

Table 4 *In vitro methods* and detection techniques used for estimating protein degradation

Method	Degradation criteria	Analyte/method	Major problems	Reference
Enzymes	Protein precipitation	N	Fungal enzyme degradation may differ from bacterial	Krishnamoorthy et al. (1983) Sniffen et al. (1992)
Rumen fluid:				
Conventional	Protein precipitation	N	Soluble proteins only	Hedqvist and Udén (2006)
	Protein precipitation	Gel electrophoresis	Laborious, measures only loss of intact proteins	Nugent et al. (1983) Messman and Weiss (1994) Spencer et al. (1988) McNabb et al. (1994)
	End-product formation	Ammonia and gas	Laborious	Raab et al (1983)
Inhibited	End-product formation	Amino acids and ammonia	Dying culture	Broderick (1987)
Whole rumen contents	Protein precipitation	N	Soluble proteins only Limited no of vessels	This paper

Figure titles:

Figure 1 Exp. 1: Recovery of TCA-precipitable N (TCA-N) after *in vitro* incubation of casein at 2 and 39°C.

Figure 2 Exp. 2 and 3: Recovery of TCA-precipitable N (TCA-N) from the first sampling time (1 min) after *in vitro* incubation at three different dosage levels of casein and egg protein with rumen contents (RC) from either a dry or a lactating cow.

Figure 3 Exp. 2: Recovery of TCA-precipitable N (TCA-N) and NH3-N concentrations at different incubation times *in vitro* at three dosage levels of casein with rumen contents (RC) from either a dry or a lactating cow.

Figure 4 Exp. 3: Recovery of TCA-precipitable N (TCA-N) and NH3-N concentrations at different incubation times *in vitro* at three dosage levels of egg protein with rumen contents (RC) from either a dry or a lactating cow.

Figure 5 Exp. 3: Disappearance of egg protein (mg TCA-N/kg rumen contents/min) *versus* dosage level (mg TCA-N/kg rumen contents) using rumen contents (RC) from either a dry or a lactating cow (n = 6) with estimates of Michaelis-Menten kinetic parameters (V_{max}=maximum rate; K_m=affinity constant).

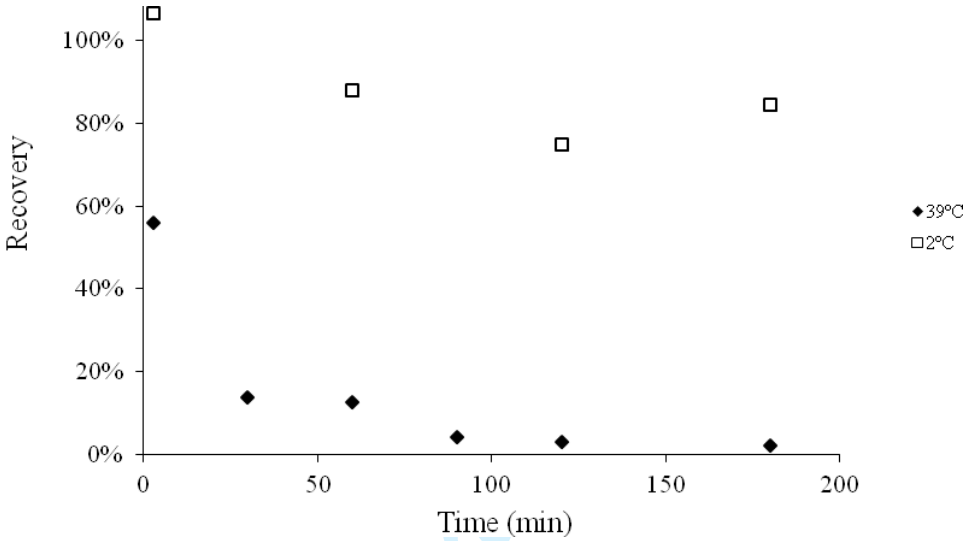

Fig. 1

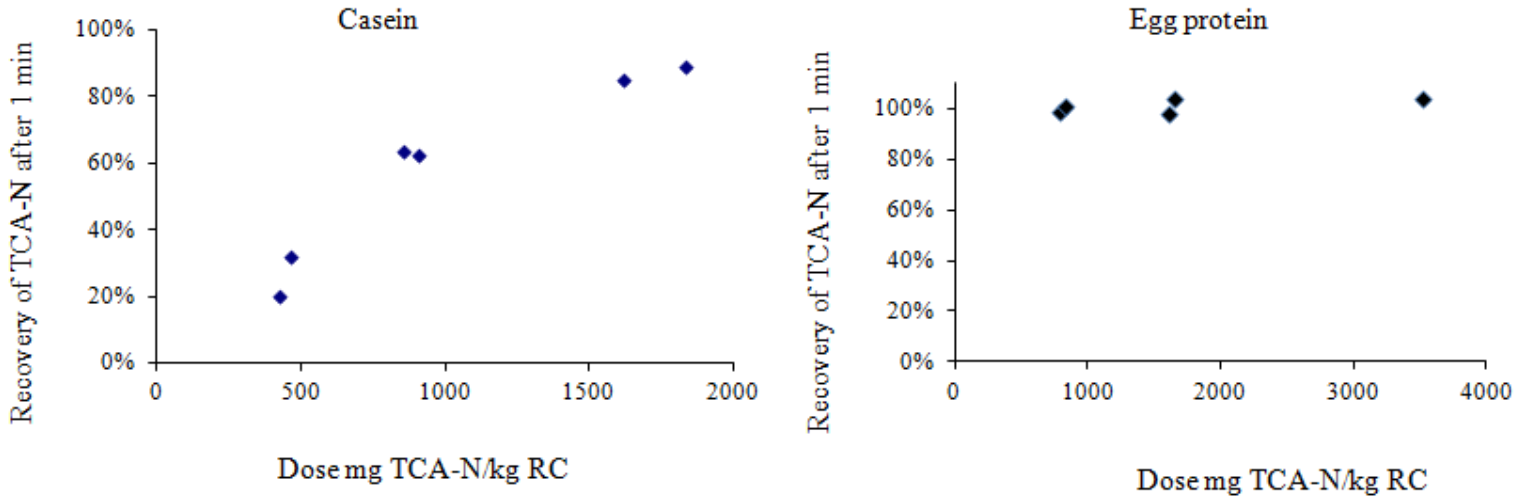

Fig. 2

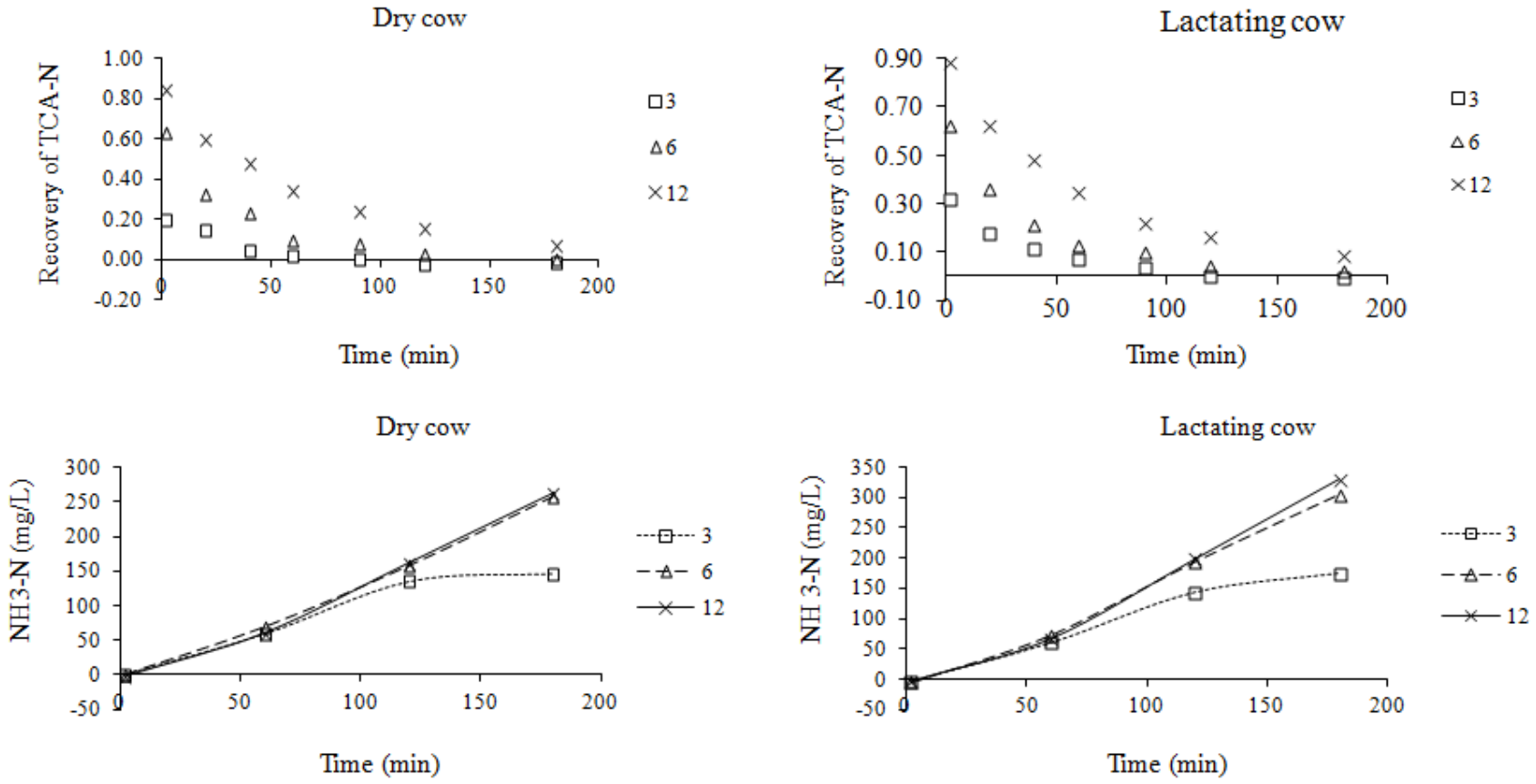
Dry cow
Recovery of TCA-N
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
0
50
100
150
200
Time (min)
3
6
12
Lactating cow
Recovery of TCA-N
0.90
0.70
0.50
0.30
0.10
-0.10
0
50
100
150
200
Time (min)
3
6
12
Dry cow
NH3-N (mg/L)
300
250
200
150
100
50
0
-50
0
50
100
150
200
Time (min)
3
6
12
Lactating cow
NH 3-N (mg/L)
350
300
250
200
150
100
50
0
-50
0
50
100
150
200
Time (min)
3
6
12

Fig. 3

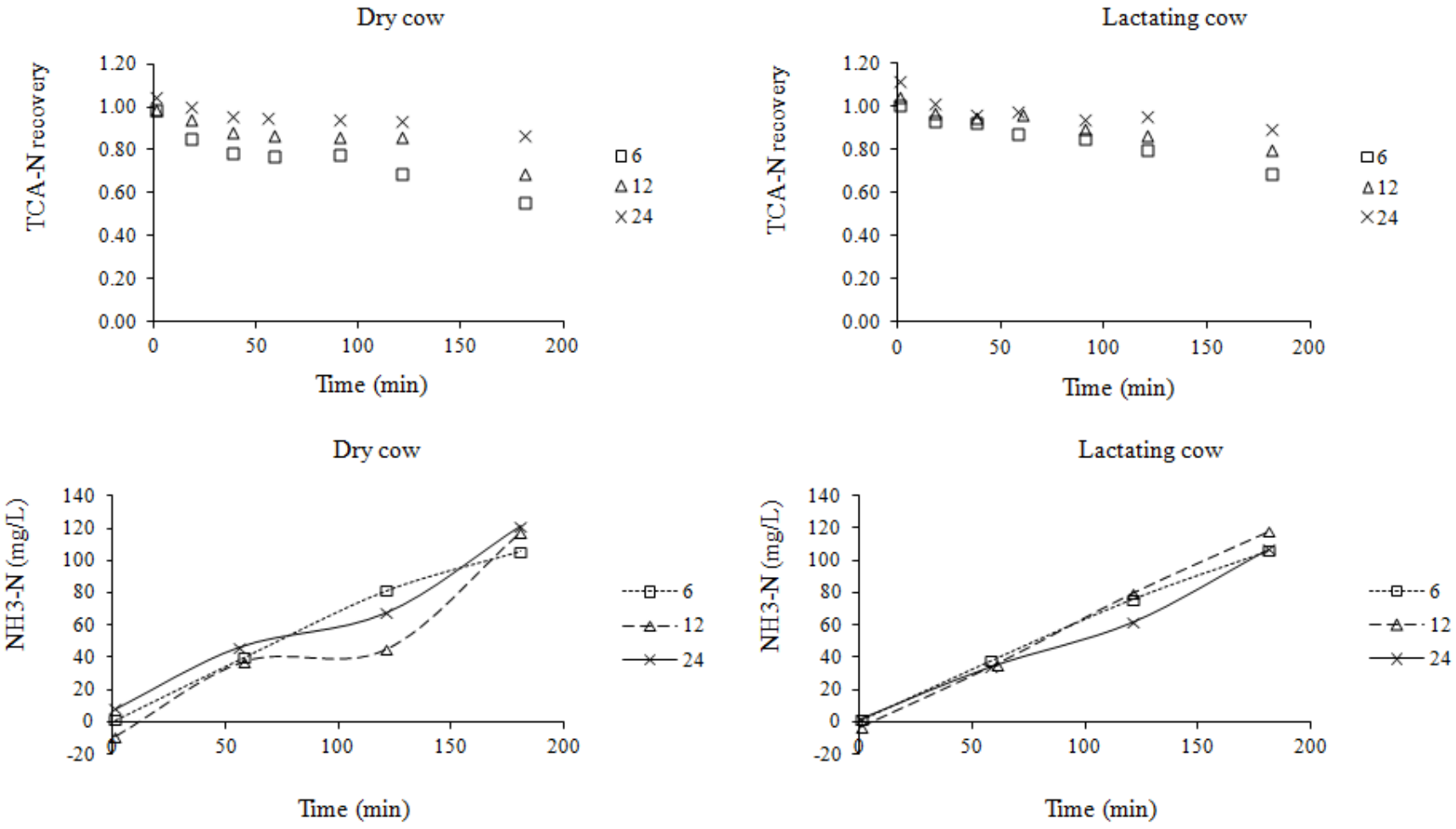
Dry cow
TCA-N recovery
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
50
100
150
200
Time (min)
6
12
24
Lactating cow
TCA-N recovery
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
50
100
150
200
Time (min)
6
12
24
Dry cow
NH3-N (mg/L)
140
120
100
80
60
40
20
0
-20
0
50
100
150
200
Time (min)
6
12
24
Lactating cow
NH3-N (mg/L)
140
120
100
80
60
40
20
0
-20
0
50
100
150
200
Time (min)
6
12
24

Fig. 4

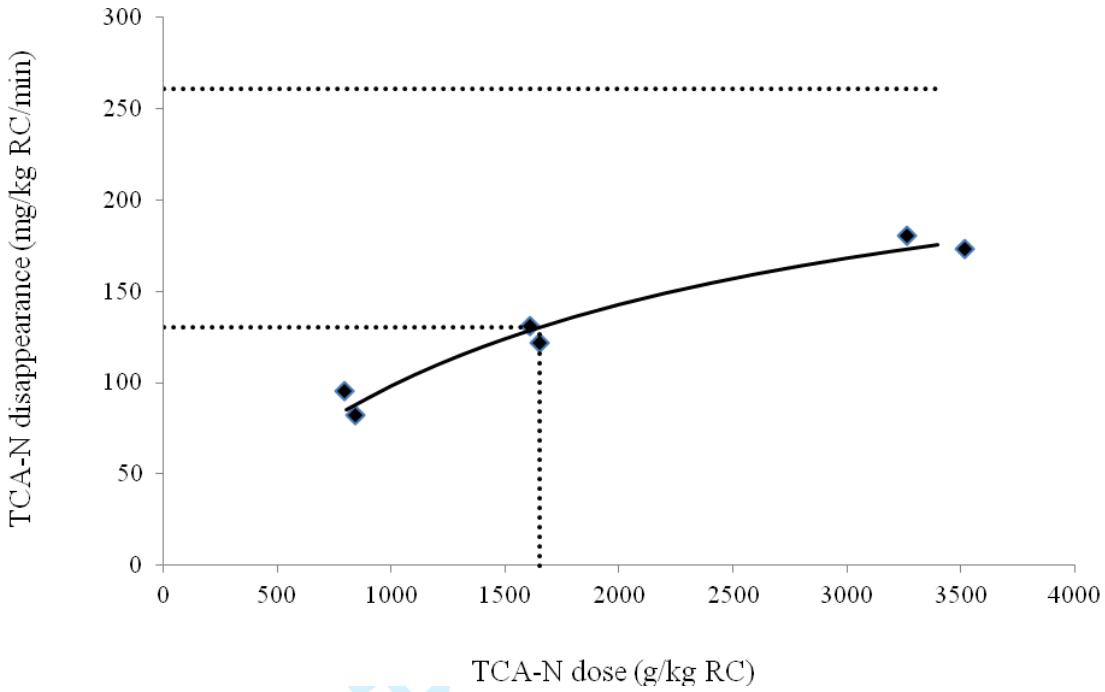

Fig. 5