THE EFFECT OF INTAKE LEVEL OF FRESH WHITE CLOVER (*TRIFOLIUM REPENS*) ON AMMONIA AND UREA KINETICS IN THE OVINE LIVER

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SUMMARY

Ureagenesis typically exceeds the rate of hepatic ammonia-nitrogen (N) extraction and it has been suggested that the shortfall in the N required for ureagenesis is compensated by amino acid (AA)-N. This study tested the hypothesis that the elevated hepatic ammonia extraction required when ruminants are fed fresh white clover (*Trifolium repens*) would require a concomitant increase in hepatic AA catabolism to supply the additional N required for ureagenesis. Our aim was to quantify the effect of 2 levels of intake (low *vs.* high) of fresh white clover on ammonia and urea kinetics in the liver. Sheep were prepared with permanent indwelling catheters in the posterior aorta via the femoral artery and in the mesenteric, portal and hepatic veins. The animals were infused for 9.5 h with $^{15}NH_4Cl$ via the cranial mesenteric vein to measure the incorporation of ^{15}N in plasma ammonia, urea and AA. For the last 3.75 h of the infusion, the infusate was supplemented with *para*-aminohippurate in order to measure hepatic blood flow. Blood samples were collected from the posterior aorta and the portal and hepatic veins to quantify the concentration of ammonia, urea and *para*-aminohippurate and the isotopic enrichment of ammonia, urea and AA in plasma. Although there were significant differences in both DM intake (807 *v.* 1118 g DM/d; s.e.d. 50.2; $P < 0.001$) and N intake (31.6 *v.* 43.9 g N/d; s.e.d. 1.96; $P < 0.001$) between the low and high groups, there were no significant differences in ammonia uptake or urea production by the liver between these groups. The hepatic ^{15}N-ammonia transfer, however, was significantly lower in the low intake group compared with the high intake group. This was not the case for the ^{15}N-urea transfers. The absence of $[^{15}N^{15}N]$ urea in plasma confirmed that ammonia contributed only one N atom to urea and, therefore, there was no evidence to suggest that elevated rates of hepatic ammonia detoxification required a disproportionate increase in AA-N catabolism for ureagenesis.

Keywords: ureagenesis, liver, intake, fresh white clover, sheep

INTRODUCTION

The New Zealand agricultural industry is based on the efficient utilisation of fresh forage, a characteristic of which is a high soluble protein content. The degradability of protein in white clover (*Trifolium repens*) is about 70-80% (Mangan 1982). Large quantities of ammonia are produced during degradation of dietary protein in the rumen (Parker *et al.* 1995) and the rate of ammonia utilisation in the rumen is often less than its rate of production, resulting in the passive absorption of excess ammonia across the rumen wall for transport to the liver (Huntington 1982). In the liver, ammonia is converted to urea, incurring an amino acid (AA) cost (Reynolds 1992; Lobley *et al.* 1995, 1996), effectively reducing the availability of AA for other purposes, such as productive gain. The hypothesis of this study was to test whether the increased rate of hepatic ammonia detoxification that would be a consequence of a high intake of fresh clover disproportionately increased the hepatic AA requirements for ureagenesis.

MATERIALS AND METHODS

The experimental procedures and protocols were reviewed and approved by the Crown Research Institute Animal Ethics Committee in Palmerston North, New Zealand according to the Animal Protection Act (1960), Animal Regulations (1987) and amendments.

Experimental animals, design and diets

Six Romney-cross wether lambs (mean initial body weight 33.4 (s.d. 0.6); 6-9 months old) were surgically fitted with silicone based catheters in the posterior aorta via the femoral artery, cranial mesenteric, portal and hepatic veins as described by Lobley *et al.* (1995). The sheep were fed fresh white clover (800 g DM/d) hourly from automatic feeders, and allowed a minimum of 4 weeks

recovery following surgery. The sheep were then (d 0 of the experimental period) offered either 800 (low intake) or 1400 (high intake) g DM/d of fresh white clover harvested daily from a pure sward with a minimum of 4-6 weeks growth. Dietary treatments were allocated according to a cross-over design. The animals were accustomed to each intake level for 10 days before a nitrogen (N) digestibility study was performed. Infusion and blood sampling occurred at d 20 of the experimental period.

Infusion and blood sampling
The sheep received a continuous infusion of $^{15}NH_4Cl$ (98.8 atom percent enrichment, Icon, Mt Marion, USA) via the cranial mesenteric vein for 9.5 h at a rate of 22.7 g/h. The $^{15}NH_4Cl$ concentration for low intake was 146 mmol/L and for high intake, 246 mmol/L. After 5.75 h of infusion, the $^{15}NH_4Cl$ infusate was supplemented with sodium *para*-aminohippurate (*p*AH; 203 mmol/L) and the infusion continued for the remaining 3.75 h.

A bolus of 5,000 IU ovine heparin was administered via a temporary catheter placed into the jugular vein. Blood was collected from the portal and hepatic veins, and from the posterior aorta, using a peristaltic pump (22.7 g/h) for the first 30 mins of each of the last 4 h of isotope infusion. The blood collection lines were passed through ice water to minimise the degradation of the blood constituents, and the blood was collected into 15 mL polypropylene vials (on ice) containing 50 µL sodium ethylenediaminetetra-acetic acid (0.15 w:v in water) as an anticoagulant.

Sample processing and analytical measurements
Collected blood (0.5 g) was deproteineised by adding 5 mL of 12% (w/w) trichloroacetic acid, mixed and centrifuged at 3,270 g for 15 mins at 4ºC. The *p*AH concentration in the whole blood was determined using the procedures described by Harris *et al.* (1992), with an additional deacetylation step (HCl; 90ºC for 65 mins).

Whole blood (2 g) was haemolysed by the addition of an equal weight of chilled L-nor-leucine (200 µmol/L) as an internal standard and 200 mL of 80 mmol/L of dithiothreitol in 0.2 mol/L phosphate buffer (pH 8.0) added. The remaining blood was centrifuged and the plasma removed and stored at -85ºC until analysed.

Plasma ammonia concentration was measured using a commercial kit (Sigma Chemical, St-Louis, USA). This kit used the reductive amination of 2-oxoglutarate in the presence of glutamate dehydrogenase. Urea concentration in plasma was determined using a commercial kit (#535, Sigma Chemical, St-Louis, USA) where the urea is converted to hydroxyl-amine in the presence of diacetylmonoxime when heated with sulphuric acid.

The isotopic enrichment of ammonia, urea and AA in haemolysed blood was determined by gas chromatography and mass spectrometry (VG Masslab LTD, Manchester, Cheshire, UK) using the methodologies described by Nieto *et al.* (1996). The isotopic enrichment of ammonia was obtained as described above after conversion of the ammonia present to nor-valine (Nieto *et al.* 1996).

Calculations and statistical analysis
Blood flow was calculated using the equations described by Katz and Bergman (1969). Hepatic arterial blood flow was calculated by difference between the splanchnic and portal venous flow. Mass transfers (or net flux) of ammonia and urea across the portal-drained viscera and the liver were calculated as described in Huntington and Reynolds (1987) and Lobley *et al.* (1995). The net hepatic ^{15}N-ammonia and ^{15}N-urea transfers were calculated as follows:

Hepatic ^{15}N transfers = $(E_h \times Z_h \times BF_h) - (E_p \times Z_p \times BF_p) - (E_a \times Z_a \times BF_a)$
where Z_h, Z_p and Z_a represent the respective concentrations in plasma, E_h, E_p and E_a were the respective isotopic enrichments, and BF_h, BF_p and BF_a were the respective blood flows in the hepatic vein (h), portal vein (p) and hepatic artery (a). Data were subjected to analysis of variance according to a cross-over design (SAS 1985) with animals treated as blocks for the effect of intake. Two animals were missing in the high intake group because of aberrant ^{15}N-urea values.

RESULTS

The DM and N intakes for the high intake group were 0.38 (807 *v.* 1118 (s.e.d. 50.2) g/d); P<0.001 and 0.39 (31.6 *v.* 43.9 (s.e.d. 1.96) g/d; P<0.001) greater than that of the low intake group.

The average portal, splanchnic and hepatic artery blood flows were similar between the low and high intake groups (Table 1). There were no significant effects of intake on ammonia or urea concentration in the portal and hepatic vein (Table 1). Consequently, the mass transfers (or net flux) across the portal-drained viscera and liver of these 2 metabolites were also similar for the 2 intake levels (Table 1).

The isotopic enrichment of urea had reached 5.9 atom percent excess (APE) after 9.5 h of $^{15}NH_4Cl$ infusion, but did not plateau (data not shown). Label recycling was evident because of the presence of ^{15}N-urea at the 3 sampling sites exceeding the rate of $^{15}NH_4Cl$ infusion (data not shown). Of the labeled urea ([$^{14}N^{15}N$] or [$^{15}N^{15}N$]) recovered across the liver, the single labeled form, [$^{14}N^{15}N$], predominated (data not shown). Double labeled [$^{15}N^{15}N$] urea was not detected in any of the blood samples. The detection limit of the GCMS was 0.2 APE.

The hepatic ^{15}N-ammonia transfer was 0.70 higher for the high intake group compared with the low intake group (Table 1). However, the ^{15}N-urea transfer was not significantly different between the 2 intake groups (Table 1). There was no detectable ^{15}N incorporation into free AA.

Table 1. Mean values and standard error of difference (s.e.d.) of blood flow, and ammonia and urea transfers, across the portal drained viscera and the liver, and ^{15}N-ammonia and ^{15}N-urea transfers across the liver of sheep fed fresh white clover at either a low (n=6) or high (n=6) intake.

Parameters	Low intake	High intake	s.e.d.	P-value
Blood flow (g/min)				
Portal vein	1746	1399	186.6	0.11
Hepatic vein	1893	1776	293.9	0.71
Hepatic artery[A]	146	376	187.2	0.27
Ammonia concentration (µmol/L)				
Portal vein	706	666	73.0	0.62
Hepatic vein	97	74	47.8	0.63
Hepatic artery[A]	96	81	29.7	0.64
Urea concentration (mmol/L)				
Portal vein	11.28	10.12	1.550	0.50
Hepatic vein	11.65	10.82	1.632	0.64
Hepatic artery[A]	11.51	10.43	1.611	0.54
Ammonia transfers (µmol/min) [B]				
Portal drained viscera	1089	823	235.0	0.31
Liver	-1090	-827	254.1	0.35
Urea transfers (mmol/min) [B]				
Portal drained viscera	-0.44	-0.50	0.52	0.91
Liver	0.69	1.23	0.36	0.17
^{15}N-ammonia transfers (µmol/min)				
Liver	-39.8	-67.8	10.58	0.03
^{15}N-urea transfers (µmol/min)[C]				
Liver	117.3	147.7	151.60	0.86

[A] The difference between the blood flow in the hepatic and in portal vein
[B] Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant tissue bed.
[C] n = 6 for low group and n= 4 for high group.

DISCUSSION

The results of the current study show that although DM and N intakes were increased by about 39% between the low and high intake groups, there was no effect of intake on net ammonia production from the portal-drained viscera and, thus, hepatic ammonia extraction and urea production were also similar between intake groups. Therefore, contrary to our hypothesis, hepatic ammonia overload did not occur with a high intake of fresh white clover. Arterial ammonia concentration (low intake - 96 *v.* high intake - 81 (s.e.d. 29.7) µmol/L, P=0.64) remained well below the metabolic overload concentration of 700 µmol/L (Symonds *et al.* 1981).

The higher ^{15}N-ammonia transfer in the high intake group compared with the low intake group was contrary to the mass transfer data where no significant difference was found. This might suggest that there was, in fact, a difference in the hepatic detoxification rate between the 2 levels of intake. It is

expected that the ^{15}N-ammonia data would be a more accurate representation of the actual ammonia status of these animals (Weijs *et al.* 1996) than the mass transfer data and, therefore, this difference truly reflects the difference in N intake between treatment groups. If this difference is real, however, it did not translate to an alteration in the ^{15}N-urea metabolism, which confirms the mass transfer values for the 2 intake groups. The relative difference between the 2 treatments was, however, much less for the ^{15}N-urea transfer than for the urea mass transfer. The absence of double labeled urea ($^{15}N^{15}N$) confirms earlier reports suggesting that the contribution of ammonia to urea-N is restricted to the single N atom (Lobley *et al.* 1995, 1996).

The absence of detectable quantities of ^{15}N in any AA is in contrast to previous findings (Lobley *et al.* 1995) and suggests that in our study, the liver detoxified ammonia using a different mechanism. Lobley *et al.* (1995) recorded significant quantities of ^{15}N incorporated into glutamine (isotopic enrichment = 3.05 APE) suggesting an increase in the activity of hepatic glutamine synthetase to remove the additional ammonia. The absence of ^{15}N in glutamine in the current study suggests that the capacity of ureagenesis was sufficient to remove the ammonia load, and that an alternative ammonia detoxification pathway (i.e. of increased glutamine synthetase activity) was not required. One striking difference between our study and that of Lobley *et al.* (1995) is the length of the period of adaptation prior to assessment of the capacity of hepatic ureagenesis. In the present study, at least 20 days of adaptation to intake was given prior to infusion of $^{15}NH_4Cl$ and assessment of the ^{15}N kinetics. It is more likely that the capacity of ureagenesis could have adapted and, therefore, increased over this period of time. This compares to a maximum of only 5 days of adaptation to the dietary regimen prior to assessment of the capacity of ureagenesis in the liver in the study published by Lobley *et al.* (1995).

The current study clearly demonstrates that significant differences in both DM and N intakes did not produce significant differences in ammonia or urea transfers across the portal-drained viscera and liver, with the sole exception of ^{15}N-ammonia transfer across the liver. There was no evidence to suggest that elevated rates of hepatic ammonia detoxification require a disproportionate increase in AA-N for ureagenesis, given sufficient time to adapt to those conditions.

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