
THE PROCEEDINGS OF THE
NEW ZEALAND SOCIETY
OF
ANIMAL PRODUCTION
VOLUME 62 2002

FREE AMINO ACIDS IN THE PLASMA OF SHEEP FED SUPPLEMENTARY PROTEIN, WITH PARTICULAR REFERENCE TO ε-N-METHYL LYSINE

I. E. B. FRASER and D. D. HADEN
Wool Research Organisation of N.Z. (Inc.), Lincoln

SUMMARY

The plasma concentration of free ε-N-methyl lysine (NML) and total free amino acids have been measured for sheep fed supplementary protein in the form of formalin-treated and untreated casein. Results from different levels of supplementation are recorded.

The addition of formalin-treated casein to a basal maintenance ration results in a significant increase in plasma free NML and total free amino acids over the levels resulting from feeding the same weight of untreated casein. Though a significant increase in free NML results from adding 30 g of casein per day to basal ration the plasma was not further significantly increased with 60 g. Changes in other free amino acids are recorded.

Changes in plasma free NML and total free amino acids are positively correlated with increasing wool weight grown when formalin-treated casein was fed.

The likely significance of NML in plasma is discussed.

AN unknown free amino acid separated during chromatography of sheep plasma was isolated, purified and identified as ε-N-methyl lysine (NML) by Weatherall and Haden (1969).

Natural occurrence of NML was first demonstrated in flagella proteins (Ambler and Rees, 1959), and later as residues in acid hydrolysates of histones from calf and rabbit thymus, liver and kidney (Murray, 1964). Recently, NML has been identified as a free acid in normal human plasma, with increased amounts in patients with serious mental disorders (Perry *et al.*, 1969). Bovine brain and plasma (Matsuoka *et al.*, 1969) and rabbit muscle protein (Glazer *et al.*, 1969) have more recently been shown to contain NML. As in human plasma, NML of bovine plasma was isolated as a free amino acid.

Work on histones has shown that NML is not incorporated into the proteins and that both L-methionine-methyl^{14}C and S-adenosyl-L-methionine-methyl-^{14}C serve as methyl donors for the methylation of the ε-amino group of L-lysine in proteins (Kim and Paik, 1965). Whilst NML has been shown to substitute for L-lysine in rats (Neuberger and Sanger, 1944) its utilization is associated with a specific demethylating enzyme, ε-alkyllysinase (Kim *et al.*, 1964). Indications are that this enzyme is active in kidney tissue (Kim *et al.*, 1963).

During preliminary studies of the free amino acids of sheep plasma undertaken by the authors, the concentration of free NML increased as a result of supplementing the basal food ration with casein. Increased plasma concentrations were observed in sheep fed formalin-treated casein as compared with untreated casein. The concentration of free NML in the plasma of sheep fed formalin-treated casein ranged from 5 to 8 moles % of the total free amino acids. Such values are far in excess of those reported for histone hydrolysates, 0.2 to 0.8 moles % (Murray, 1964). Concentration values of 0.003 μ moles/ml for plasma from healthy humans and 0.02 μ moles/ml for patients exhibiting mental disorders (Perry *et al.*, 1969) are very low compared with 0.88 μ moles/ml; the highest NML concentration recorded in plasma from the protein supplemented sheep in the preliminary studies undertaken in this laboratory.

Because of the importance of studies on administration of protein to sheep for increasing wool growth (Reis and Schinckel, 1963, 1964; Ferguson *et al.*, 1967), further study of NML and other free amino acids in sheep plasma was undertaken in relation to casein feeding and any associated increase in wool growth. Part of these studies are reported in this paper, with particular reference to NML.

MATERIALS AND METHODS

GENERAL

Blood samples for amino acid analysis were taken from two separate groups of Romney sheep.

The experimental groups fed at maintenance energy level plus supplements of 30 g and 60 g of formalin-treated casein and untreated casein per day were the sheep described by Bryden (1969), from April to September. The maintenance ration consisted of 318 g chopped hay and 545 g of whole oats per day. At the end of the August and September 28-day experimental periods, blood samples were drawn from the five sheep in each of the groups fed 30 g of formalin-treated casein and 30 g of untreated casein per day. Three sheep were sampled in the corresponding group fed 60 g of casein per day.

The experimental groups receiving three different levels of casein supplement per day (Table 3) were those groups of experimental sheep fed at maintenance energy levels described by Barry (1970), the maintenance energy level ration being 650 g chopped hay and 125 g of barley. In May, at the termination of the experimental period (Barry, 1970), plasma from the six sheep in each of the casein-supplemented groups (3 levels of supplement × 2 protein treatments) were aggregated. Amino acid analysis was carried out on each group of aggregated plasma.

Formalin treatment of the lactic casein used in all the experiments was carried out as reported by Bryden (1969).

Free Amino Acid Analysis of Plasma

Prior to daily feeding of sheep, jugular blood samples (10 ml) were collected and transferred to heparinized centrifuge tubes. The tubes were centrifuged at 12,000 rpm (Sorvell RC-2) for 20 min. The supernatant plasma was transfered to an "Amicon" pressure filter vessel (Amicon Corporation) and filtered under nitrogen at 50 lb/sq. in. through a Diaflo membrane (U.C.-3, Amicon Corporation) of 500 MW cut-off.

Amino acid analysis was carried out on the Diaflo-filtered plasma according to the technique described by Weatherall and Haden (1969). Amino acid concentrations are expressed in μm/100 ml of filtered plasma.

RESULTS

Analysis of Free Amino Acids in Plasma from Sheep Fed Formalin-treated and Untreated Casein

Analyses typifying free amino acids in plasma from sheep fed a maintenance basal ration plus 30 g/day of formalin-treated casein, and those from sheep on the same maintenance ration plus 30 g/day of untreated casein, are given in Table 1. Each amino acid value is the mean value from five sheep over the two sampling periods of August and September. The standard error refers to the variance between sheep within each protein treatment group over both sampling periods. No significant difference was detected between amino acid values for August and September.

Consistent with the preliminary studies referred to above, free NML is significantly higher in plasma concentration in the formalin-treated casein group compared with the group on untreated casein. Free valine, aspartic acid, glycine and proline are also significantly increased as a result of feeding formalin-treated casein. As a result of the increased plasma concentration of the above free amino acids there is a significantly higher total free amino acid concentration in the plasma of the formalin-treated casein group.

Relationship of ε-N-Methyl Lysine and Other Plasma Amino Acids to Different Levels of Protein Feeding

Table 2 shows the effect on free NML plasma concentration of two different levels of casein supplementation (30 g and 60 g/day) to the basal maintenance diet, together with two protein treatments.

Table 1: Free Amino Acids in Plasma from two Groups of Sheep both Fed at Maintenance and Supplemented with 30 g of Untreated Casein and 30 g of Formalin-treated Casein, respectively

Plasma Concentration in μm/100 ml ± SE based on Variance between Sheep.

Amino Acid	*Untreated Casein 30 g/day ± S.E.*	*Formalin-treated Casein 30 g/day ± S.E.*	*F*
Valine	26.6 ± 2.8	3.0 ± 3.5	10.44**
Methionine + Cystine	2.4 ± 0.3	2.4 ± 0.7	0.26 NS
Isoleucine	13.0 ± 2.1	14.4 ± 3.0	0.90 NS
Leucine	28.0 ± 1.3	28.6 ± 3.1	0.28 NS
Phenylalanine	11.3 ± 1.1	12.2 ± 2.2	1.72 NS
Histidine	7.0 ± 1.6	7.2 ± 2.4	0.03 NS
Arginine	8.0 ± 1.2	8.1 ± 2.3	0.02 NS
Lysine	13.8 ± 2.5	13.0 ± 2.8	0.39 NS
Taurine	11.1 ± 1.0	12.8 ± 3.5	2.22 NS
Aspartic Acid	20.1 ± 2.1	25.6 ± 2.3	30.88***
Threonine	22.1 ± 2.6	24.8 ± 3.2	3.32 NS
Serine	40.5 ± 3.1	40.9 ± 4.8	0.04 NS
Glutamic Acid	30.0 ± 2.6	32.8 ± 3.4	2.48 NS
Citrulline	10.0 ± 1.4	10.5 ± 1.6	0.51 NS
Proline	28.0 ± 2.6	32.3 ± 3.8	4.99*
Glycine	68.0 ± 6.2	87.8 ± 4.8	16.31***
Alanine	24.8 ± 4.5	23.1 ± 1.3	1.34 NS
Tyrosine	11.7 ± 1.5	12.6 ± 1.7	1.76 NS
Ornithine	11.4 ± 1.9	10.7 ± 2.6	0.02 NS
3-Methyl Histidine	6.9 ± 2.2	6.2 ± 1.2	0.78 NS
NML	7.6 ± 3.2	15.9 ± 5.0	19.99***
Total free amino acids	402.4 ± 20.7†	474.5 ± 26.1†	34.97***

†Differences between the totals and sum of amino acids is due to small contributions of methionine sulphoxide, methionine sulphone and amino adipic acid not included in this table.

NS Non Significant.

$*P < 0.05$. $**P < 0.005$. $***P < 0.001$.

TABLE 2: FREE NML PLASMA CONCENTRATION AS µm/100 ml ± SE BASED ON VARIANCE BETWEEN SHEEP. SHEEP FED ON MAINTENANCE RATIONS AND SUPPLEMENTED WITH TWO LEVELS OF CASEIN IN UNTREATED AND FORMALIN-TREATED FORM

	Supplement 30 g/day		*Supplement 60 g/day*	
Basal Maintenance Ration	*Untreated Casein*	*Formalin-treated Casein*	*Untreated Casein*	*Formalin-treated Casein*
4.0 ± 0.8	7.6 ± 3.2	15.9 ± 5.0	9.1 ± 2.9	22.4 ± 4.5

The feeding of untreated casein both at 30 and 60 g/day significantly increased ($P < 0.005$), free NML plasma values. No significant difference is detectable between NML values for sheep fed 30 g and those fed 60 g/day of untreated casein. The effect of formalin treatment, however, on both the 30 g and 60 g/day groups, very significantly increased free NML concentration values in plasma ($P < 0.005$) over values for the untreated casein groups.

Plasma concentration values for free NML and total free amino acids are recorded in Table 3 for three different casein supplementation levels. Plasma samples from six sheep within each casein supplement group were aggregated. Thus though analysis of the effect of casein supplementation and protein treatment on NML concentration is not possible, trends are evident in Table 3.

The concentration values in Table 3 indicate increase in NML plasma level resulting from formalin treatment of casein as compared with untreated casein. Only small dif-

TABLE 3: PLASMA CONCENTRATION OF FREE NML AND TOTAL FREE AMINO ACIDS AS µm/100 ml FROM SHEEP ON THREE DIFFERENT CASEIN SUPPLEMENT LEVELS USING BOTH UNTREATED AND FORMALIN-TREATED CASEIN

Protein Treatment	*Untreated Casein (U.T.C.)*				*Formalin-treated Casein (F.T.C.)*			
Casein supplement per day	25 g	50 g	75 g	Mean U.T.C.	25 g	50 g	75 g	Mean F.T.C.
Free NML (µm/100 ml) of plasma)	8.2	7.6	10.5	8.8	20.5	18.5	22.1	20.4
Total free amino acids (µm/100 ml of plasma)	461.2	459.0	498.0	472.7	505.0	506.1	549.8	520.3

ferences are shown in Table 3 between the NML plasma concentrations from different levels of casein supplementation within protein treatment groups. As in Table 2, results in Table 3 suggest that a much larger increase in plasma NML results from formalin treatment of protein rather than by an increase in the amount of protein supplement fed.

Total amino acid values show similar trends to NML concentrations (Table 3) in that the formalin-treated casein groups have higher blood levels than the untreated casein groups on the same level of protein supplement.

FREE NML PLASMA LEVELS AND WOOL WEIGHT

As earlier work (Ferguson *et al.*, 1967; Reis, 1969; Barry, 1969) clearly demonstrated a superior wool growth response to formalin-treated casein compared with untreated casein, NML values are plotted against wool weight grown by individual animals over 28 days of the experimental periods (Fig. 1). The wool weights are from sheep in experiments of Bryden (1969) and Barry (1970).

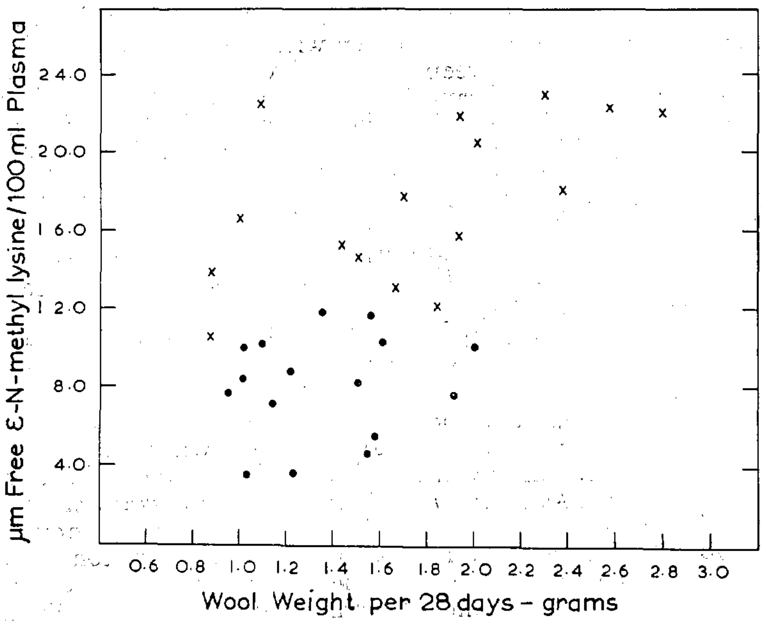


FIG. 1: *Plasma concentration of ε-N-methyl lysine compared with wool weight grown over 28 days.*

The higher plasma concentration level of free NML on formalin-treated casein supplementation as compared with untreated casein is clearly demonstrated in Fig. 1. There is a highly significant linear function of NML concentration and wool weight ($r = 0.78$, $P < 0.001$). Similarly, the values of NML are a linear function of total amino acids minus N-methyl lysine ($r = 0.68$, $P < 0.001$). The total amino acids also show a significant relationship to wool weight ($r = 0.44$, $P < 0.025$).

The other free amino acids, glycine, aspartic acid and valine which individually significantly increase in plasma concentration (Table 1) show no significant linear relationship to wool weight produced.

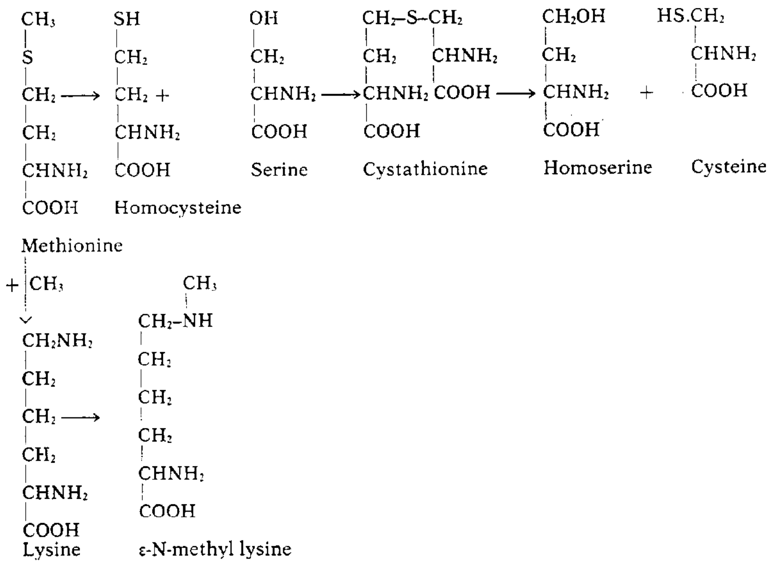
DISCUSSION

As NML has been identified as a natural constituent in sheep plasma, and as an increase in plasma level results from feeding untreated casein, the higher level recorded in the plasma of sheep fed formalin-treated casein is not likely to result as a direct effect *in vivo* of formalin on lysine.

The significant increase in plasma NML after feeding formalin-treated casein, together with the positive, significant relationship between NML and wool weight has provided preliminary information on the role of NML. Further trials are needed to confirm the reported effects, and consideration of likely biochemical pathways involving NML is warranted. Other individual free amino acids, which likewise increase in plasma concentration on feeding formalin-treated casein, have not been shown to correlate with increased wool weight. Total free amino acids, minus free NML, show a significant linear increase with increasing NML and wool weight.

Several possible pathways of metabolism may be associated with an increase in NML together with increased wool weight.

A possible explanation for the presence of NML in plasma free amino acids, based on known biochemical pathways, is the methylation of lysine by the methyl group of methionine. As methionine is the most important source of labile methyl and is the major primary methyl donor in transmethylation, together with the demonstration that L-methionine and S-adenosyl methionine do methylate ε-amino groups of lysine (Murray, 1964; Kim and Paik, 1965) important precedents are available. The resultant formation of homocystine is the likely initiator of a second pathway (Berg, 1953) leading to the formation of cyst(e)ine which would provide a more direct association with known specific requirements for wool growth, namely, cyst(e)ine being limiting for wool growth. Daily supplements of cystine and methionine have been shown to increase wool growth by as much as 130% and increase wool sulphur content by 24 to 35% (Reis and Schinckel, 1963). The suggested metabolic pathways are as follows:



In the above scheme NML is a by-product (of indirect significance) yet a direct indication of homocystine formation, and cyst(e)ine availability. The use of NML plasma level as an index of wool growth stimulation through increased cyst(e)ine availability would be effected by variations in the transthiolation steps and the demethylation and/or excretion of NML.

The significantly increased plasma level of free amino acids when formalin-treated casein is fed is due, presumably, to a higher concentration of amino acids being absorbed from the intestine when such casein is fed as compared with untreated casein. Such plasma amino acid increase may be as important as any other factor in promoting greater wool growth.

ACKNOWLEDGEMENTS

To Professor A. E. Henderson, Lincoln College, and Dr T. N. Barry, Invermay Agricultural Research Centre, for making sheep available for plasma studies, and permission to use some wool weight values.

REFERENCES

Ambler, R. P.; Rees, M. W., 1959: *Nature* (Lond.), *184*: 56.
Barry, T. N., 1969: *Proc. N.Z. Soc. Anim. Prod., 29*: 218.
———, 1970: *Proc. N.Z. Soc. Anim. Prod., 30*: 216.
Berg, C. P., 1953: *Physiol. Rev., 32*: 145.
Bryden, J. McG., 1969: M.Agr.Sc. Thesis, Lincoln College, University of Canterbury.
Ferguson, K. A.; Hemsley, J. A.; Reis, P. J., 1967: *Aust. J. Sci., 30*: 215.
Glazer, A. N.; De Lange, R. J.; Martinez, R. J., 1969: *Biochim. Biophys. Acta, 188*: 174.
Kim, S.; Benoiton, L.; Paik, W. K., 1963: *Biochim. Biophys. Acta., 71*: 745.
Kim, S.; Benoiton, L.; Paik, W. K., 1964: *J. biol. Chem., 239*: 3790.
Kim, S.; Paik, W. K., 1965: *J. biol. Chem., 240*: 4629.
Matsuoka, Y.; Kumon, A.; Nakajima, T.; Kakimoto, Y.; Sano, I., 1969: *Biochim. Biophys. Acta., 192*: 136.
Murray, K., 1964: *Biochemistry, 3*: 10.
Neuberger, A.; Sanger, F., 1944: *Biochem. J., 38*: 119.
Perry, T. L.; Diamond, S.; Hansen, S., 1969: *Nature* (Lond.), *222*: 668.
Reis, P. J.; Schinckel, P. G., 1963: *Aust. J. biol. Sci., 16*: 218.
———; ———, 1964: *Aust. J. biol. Sci., 17*: 532.
Reis, P. J., 1969: *Aust. J. biol. Sci., 22*: 745.
Weatherall, I. W.; Haden, D., 1969: *Biochim. Biophys. Acta., 192*: 553.