Effect of diet and infusion of volatile fatty acids into the rumen on the concentration of plasma free amino acids in sheep

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(*Received 26 October 1981 – Accepted 31 March 1982*)

1. The effect of supplementing barley diets with urea (U), extracted decorticated groundnut meal (GNM) or Peruvian fish meal (PFM) on plasma free amino acid concentrations in sheep have been examined and the first limiting amino acid has been indicated by measuring the changes in the concentration of the plasma essential amino acids (PEAA) during a rumen infusion of a volatile fatty acid (VFA) mixture.

2. Three wethers fitted with rumen and re-entrant duodenal cannulas were given isonitrogenous, isoenergetic diets containing (g/kg dry matter (DM)) U 20, GNM 106 or PFM 78, the crude protein (nitrogen × 6·25) contents being 139, 145 and 148 respectively. The sheep were fed hourly, the mean daily DM intake being 0·634 kg.

3. Plasma concentrations of valine, threonine, lysine, isoleucine and leucine were linearly related to their concentrations in duodenal digesta.

4. A VFA mixture was infused into the rumen for 6 h to supply (mmol/min) acetate 1·47, propionate 0·22 and *n*-butyrate 0·27. Blood samples were taken 6 h before, during and 12 h after the end of the infusion.

5. The concentration of all PEAA decreased relative to the pre-infusion and post-infusion controls but there were no significant differences between diets.

6. The mean decreases in concentration averaged over all three diets showed that the decrease in concentration of methionine (41·5%) was far greater than for any other essentia' amino acid suggesting that under these conditions methionine was the first limiting amino acid.

The postprandial changes in jugular blood plasma free amino acid (PAA) concentrations have been shown in single-stomached animals to reflect the amino acid composition of the diet (Denton *et al.* 1953) and have been used to determine the order of limiting amino acids in diets fed to dogs (Longenecker & Hause, 1959), rats (Hill & Olsen, 1963) and pigs (Windels *et al.* 1971). However, in ruminant animals the degradation of dietary protein and synthesis of microbial protein in the rumen modifies the pattern of amino acids presented to the small intestine for absorption and, therefore, attempts to relate dietary amino acid composition to plasma levels have yielded equivocal results (Schelling *et al.* 1967; Burris, Bradley *et al.* 1974). In the experiment now described, we have investigated to what extent PAA were related to the pattern of amino acids presented to the duodenum when sheep were given isoenergetic, isonitrogenous barley-based diets containing supplements of urea (U), extracted, decorticated groundnut meal (GNM) or Peruvian fish meal (PFM).

The sequence of limiting amino acids in the diet for single-stomached animals has been determined by comparing the postprandial PAA with a reference pattern obtained either after fasting (Longenecker & Hause, 1959) or feeding a non-protein diet (Hill & Olsen, 1963) or feeding a reference protein (Smith & Scott, 1965). These techniques are not applicable to the ruminant animal if normal rumen digestion is to be maintained. We have used an alternative approach based on the relative depressions in plasma essential amino acid (PEAA) concentrations resulting from increased energy supply. This effect was first shown in man (Munro & Thompson, 1953; Swendseid *et al.* 1967) where ingestion of glucose leads to a decrease in PEAA concentrations due to a stimulation of uptake of amino acids from the plasma by tissues and an apparent increase in tissue protein synthesis. The application of this effect to the determination of the limiting amino acid assumes that the depressions

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in PEAA reflect amino acid requirements and that the amino acid undergoing the greatest relative depression is first limiting. This method has been applied to ruminants using arterial or venous infusions of glucose or propionate (Potter *et al.* 1968; Potter *et al.* 1972; Eskeland *et al.* 1974; Faichney, 1974; Offer *et al.* 1975). However, Potter *et al.* (1972) reported that the decline in PEAA after glucose infusion in both sheep and rats did not correctly predict the first limiting amino acid. Since the arterial or venous infusion of glucose in ruminants may cause unphysiological responses in insulin, we have examined the effect of a rumen infusion of a mixture of volatile fatty acids (VFA) on PEAA in sheep to determine whether such changes might be used to predict the sequence of limiting amino acids for protein synthesis in the ruminant.

EXPERIMENTAL

Sheep, diets, design and collection of duodenal digesta

This experiment was a continuation, within each sheep-period, of the experiment described by Mercer *et al.* (1980). Details of the sheep, diets, experimental design and collection of duodenal digesta were also described by Mercer *et al.* (1980). In each period, each sheep was fitted with a catheter into a jugular vein immediately after the final collection of duodenal digesta. On the following day, plasma samples were taken before, during and after infusion of a mixture of VFA into the rumen. The sheep continued to receive their respective diets automatically every 60 min throughout the experiment and were allowed free access to water.

VFA infusion and blood sampling

The infusate contained acetic, propionic and *n*-butyric acids in the molar proportions of 0·75, 0·11 and 0·14 respectively and was infused to supply 1·47, 0·22 and 0·27 mmol acid/min respectively.The acids were in aqueous solution, with the pH adjusted to 5·0 with a solution containing 1 M-sodium hydroxide, 1 M-potassium hydroxide and 0·5 M-calcium hydroxide. The VFA proportions were based on those determined in rumen fluid from the first sheep used in the experiment. The gross energy infused was equivalent to a rate of 5·56 MJ/24 h. This was calculated to be approximately equal to the supply of energy, as VFA, from fermentation of the diets. The infusion was preceded by a primer injection of 35 ml of the infusate into the rumen. The solution was then infused at 35 ml/h using a peristaltic pump (Perpex 10200, LKB Instruments Ltd, South Croydon, Surrey) for 6 h.

Jugular blood (approximately 10 ml) was taken into an heparinized syringe at 08.30 hours, 6 h before the commencement of the infusion (−6 h). Further samples were taken at 4, 5 and 6 h (+4, +5 and +6 h) during the infusion. A final sample was taken the following morning 12 h after the end of the infusion (+18 h). The blood was centrifuged immediately for 10 min at 4° at 3000 *g* and the plasma treated by the method of Schelling *et al.* (1967) with the following modifications: norleucine was added as an internal standard before the picric acid solution; the excess picric acid was removed on a 40 × 10 mm column of AG2-X8 (200–400 mesh) analytical grade anion-exchange resin (Cal Biochem. Ltd, London); after adjustment of the pH to 2·0 with 1 M-hydrochloric acid the solution was evaporated under reduced pressure almost to dryness, taken up in approximately 3 ml water, freeze-dried and then stored at −20° until analysed.

PAA analysis

The concentration of PAA was determined by the use of an amino acid AutoAnalyzer (Technicon Instruments Co. Ltd, Basingstoke, Hants). A sodium citrate buffer gradient system incorporating an Autograd was used and the 1400 × 6 mm column was Chromobeads Type A (Technicon Instruments Co. Ltd). The column was operated at 60°, a buffer flow-rate of 30 ml/h and a ninhydrin flow-rate of 66 ml/h. The freeze-dried salts of the PAA were taken

up in citrate buffer, pH 2·00, and made up to 5 ml. (The +4, +5 and +6 h samples were combined and made up to a volume of 15 ml.) A portion (1 ml) of this solution, equivalent to 1 ml plasma was applied to the column. Separate analyses of the +4, +5 and +6 h samples from three experiments did not reveal any consistent linear or quadratic changes in the concentration of any amino acid. Threonine and serine were well separated with no peak in the expected position for asparagine and glutamine. Others, using the same picric acid deproteinization technique, have not reported values for asparagine and glutamine (Theurer *et al.* 1966; Schelling *et al.* 1967; Hogan *et al.* 1968) and glutamine is known to be labile under these conditions of sample preparation (Stein & Moore, 1954). Consequently the glutamic acid peak may contain a contribution from glutamine. Peaks in the expected positions of citrulline and ornithine were observed but these were not estimated quantitatively. Methionine was clearly separated between cystine and isoleucine. A small peak was observed following and clearly separated from methionine. This was neither identified nor estimated quantitatively. Tryptophan was not detected as some of it is precipitated bound to the plasma proteins (Ohara & Ariyoshi, 1979) and much of the remainder is adsorbed on the AG2-X8 resin during picric acid removal (Stein & Moore, 1954).

Statistical analysis

Analysis of variance was carried out with complete partitioning of degrees of freedom. The error mean square was obtained by summation of the appropriate interaction containing the random variable (sheep) according to the procedure described by Sokal & Rohlf (1969). For the effect of diet on PAA concentration only the values from the control samples (−6 h and +18 h) were used. In the analysis, 1 degree of freedom (df) was allocated to time; sheep × time interactions were included with sheep × diet interactions in the error term only when they were not significantly different according to the pooling procedure described by Sokal & Rohlf (1969). For the effect of energy infusion on PAA concentration all values were statistically examined for linear and quadratic components varying with time, the assumption being that if the VFA infusate had been without effect the concentration of each amino acid in the pre-, during and post-infusion periods would have been related in a linear manner. The effect of energy infusion was determined as the quadratic component of time and was tested against sheep × time quadratic (2 df) alone or combined with sheep × diet × time quadratic (4 df) as the error term. When the F test was significant, the dietary means were tested by Duncan's multiple range test as modified by Harter (1960).

The regressions of amino acid concentrations in plasma on those in duodenal digesta were carried out both on a within-sheep basis, with pooling of the regression coefficients for the three sheep and calculation of the error term with 2 df from the variation of the regression slopes between sheep, and by ignoring the classification by sheep, regarding the nine pairs of values for each amino acid as independent, giving 7 df for testing linear relationships. The regression coefficients were similar by the two methods but fewer achieved statistical significance because of the few degrees of freedom by the more exacting method.

RESULTS

Effect of diet on PAA concentration

The PAA concentrations in the control samples for each diet are shown in Table 1. The PEAA concentrations were generally greater on the PFM diet than on either of the other two diets, but the differences only achieved statistical significance for lysine, histidine and cystine. There was little difference between the PEAA concentrations on the GNM and U diets except for a lower cystine concentration on the GNM diet. The non-essential amino acids showed no consistent pattern of response to diet.

Table 1. *Effect of urea (U), groundnut meal (GNM) or Peruvian fish meal (PFM) supplements in isonitrogenous, isoenergetic diets on the concentration (μmol/l) of plasma free amino acids*
(Mean values with their standard errors)

Amino acid	Diet			SEM	df	Statistical significance of difference between diets		
	U	GNM	PFM			PFM v. U	PFM v. GNM	GNM v. U
Aspartic acid	12·0	9·8	9·8	1·20	10	NS	NS	NS
Threonine	107·5	153·7	158·7	20·99	4	NS	NS	NS
Serine	66·6	88·5	68·5	11·61	4	NS	NS	NS
Glutamic acid	127·8	110·8	100·6	7·48	10	*	NS	NS
Proline	86·0	76·5	79·1	7·65	10	NS	NS	NS
Glycine	548·6	458·1	567·2	22·24	4	NS	*	*
Alanine	154·9	167·2	121·2	5·05	10	*	***	NS
Valine	167·4	169·9	210·1	13·41	10	†	†	NS
Cystine	39·5	31·6	45·8	1·50	10	•	***	**
Methionine	14·1	16·1	20·1	1·88	10	†	NS	NS
Isoleucine	88·4	89·9	102·9	7·01	8	NS	NS	NS
Leucine	91·5	106·7	115·1	9·30	10	NS	NS	NS
Tyrosine	44·7	49·7	49·1	3·42	10	NS	NS	NS
Phenylalanine	44·8	44·8	43·0	2·85	10	NS	NS	NS
Lysine	136·1	143·0	190·8	15·39	6	†	†	NS
Histidine	116·6	111·5	146·3	5·80	6	*	**	NS
Arginine	71·8	74·1	94·1	5·91	6	*	†	NS

† $P < 0·1$, * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
NS, not significant, $P > 0·10$.

Effect of a rumen infusion of VFA on PAA concentration

The decreases in PAA concentration during the infusion compared to the mean of pre- and post-infusion (−6 h and +18 h) are given in Table 2. Of the non-essential amino acids, only the concentration of glycine decreased significantly and this decrease was greater ($P < 0·05$) on the U diet than on either the GNM or PFM diets. The concentrations of all the PEAA with the exception of histidine decreased. The decreases (%) averaged over the three diets were: methionine 41·5, isoleucine 16·3, lysine 15·4, valine 15·0, leucine 14·7, phenylalanine 13·7, threonine 11·1. The concentration of cystine decreased on the PFM diet but not on either the GNM or U diets ($P < 0·005$) so that the change averaged over all three diets was not significant. No other significant differences in response to the different diets and energy infusion were obtained.

DISCUSSION

Relationship of dietary and duodenal amino acid supply to PAA concentration

Because of extensive degradation of dietary protein in the rumen, poor relationships have consistently been observed between dietary amino acid composition and PAA concentration (Schelling *et al.* 1967; Williams & Smith, 1974).

However, PAA concentration is influenced by the proportions of amino acids presented to or absorbed from the small intestine. For example, infusion into the abomasum or duodenum of a single essential amino acid increases the plasma concentration of that amino acid (Wakeling *et al.* 1970; Tao *et al.* 1974; Richardson & Hatfield, 1978). Infusion of casein

Table 2. *Decrease‡ in concentration (μmol/l) of plasma amino acids when a mixture of volatile fatty acids was infused into the rumen of sheep given isonitrogenous, isoenergetic diets containing supplements of urea (U), groundnut meal (GNM) and Peruvian fish meal (PFM)*

(Mean values with their standard errors)

Amino acid	Diet			SEM	All diets	SEM	df	Statistical significance of decrease (all diets)
	U	GNM	PFM					
Aspartic acid	−0·8	0·6	1·7	1·62	0·5	0·94	6	NS
Threonine	8·0	22·8	15·4	7·26	15·5	4·19	6	*
Serine	5·8	5·7	−2·2	4·77	3·1	2·75	6	NS
Glutamic acid	−20·9	15·4	−2·5	9·62	−2·7	5·55	6	NS
Proline	10·4	7·1	5·7	10·04	7·8	5·80	6	NS
Glycine	34·9[a]	7·6[b]	−1·1[b]	6·68	—	—	6	—
Alanine	23·5	16·6	11·4	17·79	17·1	10·27	6	NS
Valine	23·0	41·1	17·9	10·21	27·3	5·90	6	**
Cystine	−4·1[a]	−1·5[a]	9·6[b]	1·63	—	—	6	—
Methionine	5·5	7·6	7·7	1·07	6·9	0·62	6	***
Isoleucine	11·3	22·9	11·7	8·07	15·3	4·66	6	*
Leucine	8·5	23·1	14·5	8·33	15·4	4·81	6	*
Tyrosine	5·9	7·5	2·8	4·34	5·4	2·50	6	†
Phenylalanine	6·7	8·6	3·0	2·67	6·1	1·54	2	†
Lysine	15·1	29·7	27·8	7·56	24·2	4·36	6	**
Histidine	10·7	−3·4	−0·3	12·94	2·3	7·47	2	NS
Arginine	−16·8	3·4	7·6	10·87	−1·9	6·28	6	NS

† $P < 0{\cdot}10$. * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

‡ $\text{Decrease} = \left(\frac{\text{pre-infusion} + \text{post-infusion concentration}}{2}\right) - \text{infusion concentration}.$

NS, not significant, $P > 0{\cdot}10$.

[a, b] Values with the same superscript letter in the same horizontal row were not significantly different ($P > 0{\cdot}05$).

into the abomasum and formaldehyde treatment of dietary proteins, which increases the flow of amino acids to the abomasum, also result in increased PEAA concentrations in sheep (Hogan *et al.* 1968; Faichney, 1974) and in lactating dairy cows (Broderick *et al.* 1974). The present study confirms that similar relationships can be detected when sheep are given normal dietary proteins which result in relatively smaller changes in the pattern of amino acids presented to the duodenum. Table 3 shows some linear relationships between the concentration of amino acids in duodenal digesta and plasma which were calculated without taking into account any sheep effects. The plasma concentration of threonine, valine, isoleucine and lysine increased with increase in duodenal concentration. When analysed on a within-sheep basis, plasma leucine also increased with duodenal concentration. However, in agreement with the conclusion of Hogan *et al.* (1968), the variation in the relationships was such that measurement of plasma amino acid concentration is unlikely to be of use in predicting the flow of amino acids to the duodenum.

The generally higher PEAA concentrations on the PFM diet, therefore, may be interpreted as additional evidence that a high proportion of PFM passed through the rumen undegraded, while the similarity of values for U and GNM diets is in accord with the extensive degradation of GNM (Mercer *et al.* 1980). In particular, the increased plasma lysine concentration may reflect the higher lysine content of the PFM compared to the GNM or U diets. However, plasma histidine and cystine were also significantly increased on the PFM diet despite the fact that the concentrations of these two amino acids in PFM (Miller,

Table 3. *Regression equations relating plasma free amino concentration* (Y, *μmol/l*) *and duodenal amino acid concentration* (X, *g/kg nitrogen*)

Amino acid	Regression equation	Correlation coefficient	Residual standard deviation	Statistical significance
Valine	$Y = 1{\cdot}21\ X - 236{\cdot}9$	0·85	27·7	**
Threonine	$Y = 1{\cdot}17\ X - 269{\cdot}7$	0·81	32·8	**
Lysine	$Y = 0{\cdot}49\ X - 64{\cdot}1$	0·75	28·7	*
Isoleucine	$Y = 0{\cdot}51\ X - 75{\cdot}5$	0·70	16·0	*
Leucine	$Y = 0{\cdot}34\ X - 34{\cdot}8$	0·64	24·8	†
Phenylalanine	$Y = 0{\cdot}25\ X - 38{\cdot}0$	0·55	7·6	NS
Methionine	$Y = 0{\cdot}10\ X - 0{\cdot}8$	0·53	5·0	NS

† $P < 0{\cdot}10$, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.
NS, not significant, $P > 0{\cdot}10$.

1970) are no higher than in GNM (Pion & Fauconneau, 1966), barley (FAO, 1970) or rumen bacteria (Purser & Buechler, 1966). In the instance of cystine this may reflect the increased supply of methionine with the PFM diet, since Nimrick *et al.* (1970) observed increased plasma cystine concentrations following methionine infusion and Radcliffe & Egan (1974) have shown the key enzymes of the transulphuration pathway to be present in the liver and kidney of ruminants. Bergen *et al.* (1973) and Burris, Boling *et al.* (1974) have also observed higher PAA levels in sheep and steers given fish meal-supplemented diets.

Effect of a rumen infusion of VFA on PEAA concentrations

This experiment clearly demonstrates the effect of energy supply from the rumen on PEAA under conditions of hourly feeding when the supply of amino acids for absorption can be expected to be reasonably constant. The VFA infusate was based on the relative molar proportions present in rumen fluid from the first sheep started on the experiment with the object of increasing the supply of energy without appreciably altering the relative molar VFA proportions. Unfortunately this proved not to be representative of the average proportions throughout the experiment (Mercer *et al.* 1980). Subsequent to this experiment, Eskeland *et al.* (1974) showed venous infusions of acetate and butyrate were less effective than propionate or glucose in depressing PEAA concentrations. However, despite the low propionate content of the VFA mixture, the rumen infusion in the present experiment depressed the PEAA concentration appreciably. Therefore, the variation in PEAA after a single meal can be expected to reflect both changes in amino acid supply to the duodenum and rate of energy supply from fermentation in the rumen. Differences in these two factors might account for the different responses reported in the literature (see Williams & Smith, 1974). For example, the difference in response of PEAA after a feed of concentrates compared to roughages is probably best explained in terms of energy supply (Purser *et al.* 1966).

If it is assumed that the depressions in PEAA concentrations are brought about by a short-term cellular uptake of amino acids in response to the greater energy supply and that cellular uptake is proportional to cellular needs for metabolism and protein synthesis, then the changes induced by VFA infusion enable an assessment of the limiting order of essential amino acids. In this experiment plasma methionine decreased most when energy supply was increased irrespective of diet. Thus, although the PFM diet supplied greater quantities of the essential amino acids, including methionine, to the duodenum, the ratio, methionine: other essential amino acids apparently remained below the optimum for tissue metabolism

and protein synthesis. This may result from the high requirement for sulphur-containing amino acids for wool growth, and accords with the observations of Reis (1967) that infusions of methionine or cystine to the abomasum increased wool growth by as much as 100%. The percentage decreases in the concentration of the other essential amino acids were closely similar and no conclusion can be reached on the identity of the possible second or third limiting amino acids.

Offer *et al.* (1975) observed plasma tryptophan to be most reduced by glucose infusion in pre-parturient but not post-parturient ewes. Tryptophan concentration in plasma was not determined in the present study nor in other previous studies. However, the sequence of amino acids most affected by glucose infusion in the work of Offer *et al.* (1975) was dependent on whether or not the saline control values were included in the calculation. Eskeland *et al.* (1974) also found methionine to be decreased by a greater proportion than lysine, arginine, isoleucine and leucine following venous infusion of glucose, acetate, propionate or butyrate to lambs receiving a high-concentrate diet. However, when lambs or sheep were given high-roughage diets, the infusion of glucose or propionate reduced plasma methionine concentration by an extent similar to that of the other four amino acids (Potter *et al.* 1972; Eskeland *et al.* 1974). If these techniques correctly predict the first and subsequent limiting amino acids, then no one amino acid is consistently more limiting than others, but the sequence is affected by factors such as diet, energy supply and physiological status of the animal.

Acknowledgement is made to the Agricultural Research Council for a grant and postgraduate scholarship held by J. R. Mercer.

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Printed in Great Britain