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Amino-acid Metabolism in the Rumen of the Sheep

By D. LEWIS

Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

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During recent years considerable attention has been given to the breakdown of protein in the rumen, and in particular to ammonia production during this process. The first suggestion of the significance of ammonia in the rumen was made by Pearson & Smith (1943) based on the finding that ammonia was produced in vitro during the incubation of rumen contents with protein. McDonald (1948, 1952) recognized the importance of ammonia in the rumen and studied the changes in the ammonia concentration in rumen contents following the feeding of various proteins: he demonstrated the production of ammonia in vivo from ingested protein and also the absorption of significant quantities of ammonia from the rumen. He suggested that urea derived from this ammonia in the liver would partly be excreted and partly returned to the rumen in the saliva.

It seems probable that some of the ingested protein is converted to amino-acids which are deaminated giving rise to ammonia. However, it is not known in fact that amino-acids are intermediates in this reaction, neither have they been shown to be present in rumen contents. Using washed suspensions of rumen micro-organisms El-Shazly (1952*a*, *b*) demonstrated that casein hydrolysate was converted to ammonia, carbon dioxide and volatile fatty acids. The extent to which deamination occurred was found to vary with the diet of the animal; as the protein in the diet was raised so the rate of deamination by the washed suspensions increased, probably owing to an increase in the number of micro-organisms capable of attacking amino-acids. A detailed examination was not made of the products formed during the breakdown of individual amino-acids.

The present work has been concerned with an extension of these findings and in particular with the breakdown of amino-acids in the rumen and by washed suspensions of rumen micro-organisms in vitro. It was first necessary, however, to determine whether amino-acids were to be found in rumen contents.

EXPERIMENTAL AND RESULTS

Animals and their management. Three Clun Forest sheep fitted with permanent rumen cannulas were used and fed on different rations. Sheep no. 24 was given 900 g hay (8·3% protein) daily; sheep no. 8, 700 g hay and 150 g casein daily; and sheep no. 12, 400 g hay with 500 g dried grass (18·2% protein) daily. The food was given at 9 a.m. and any excess was removed 2 h later. The weights of the animals were about 50 kg and they had free access to water and mineral licks. The animals were fed on these diets for at least 4 weeks before the experiments began and were trained to eat at least 90% of the food within 2 h. Samples of rumen contents were withdrawn by gentle suction through the cannula. The doses of amino-acids or casein (see p. 218) given were inserted into the rumen through the cannula.

Washed suspensions of rumen bacteria were prepared according to the method of Sijpesteijn & Elsden (1952). The sample of rumen contents was withdrawn 2–2·5 h after the food was removed; unless otherwise stated sheep no. 8 fed on hay with a casein supplement was used.

Volatile fatty acids. Samples of rumen contents, after filtering through muslin, or samples of the contents of manometer vessels, were made up to 3 ml. and acidified with 1 ml. 5N-H_2SO_4. The volatile fatty acids were separated by steam distillation in a Markham (1942) apparatus and two successive volumes of 75 ml. of distillate were collected, the second serving as a control. The distillates were aerated with CO_2-free air for 3 min and then titrated with alkali using phenolphthalein as an indicator under CO_2-free conditions. The identification and estimation of individual fatty acids were made by the method of James & Martin (1952) with modifications (Annison, 1954).

Estimation of amino-acids. Amino-N was determined by measuring the N liberated on treatment with HNO_2 in the Van Slyke-Neill manometric apparatus (Peters & Van Slyke, 1932). Chromatographic examination of amino-acids was made using collidine saturated with water, *n*-butanol saturated with 20% (v/v) acetic acid and phenol-ammonia-nitrogen as developing solvents.

Other analytical methods. Manometric methods used have been described previously (Lewis, 1954). Ammonia was determined by the method of Conway (1947) using potassium metaborate instead of potassium carbonate in the outer chamber of the Conway unit. Lactic acid was estimated by the method of Friedemann & Graeser (1933), and ketone bodies as described by Greenberg & Lester (1944).

Examination of rumen contents for free amino-acids

It is usually considered that the quantity of free amino-acids in rumen contents is very small, even if they are present at all. MacDonald (1952) could not detect amino-acids qualitatively by direct ninhydrin test or by paper chromatography of protein-free filtrates. Therefore a procedure was developed to concentrate rumen contents after the removal of food particles and micro-organisms. An attempt was made to detect and estimate any amino-acids present in this concentrate after desalting and removing the ammonia. The amino-acids were examined by paper chromato-

graphy and the Van-Slyke amino-N method. To avoid losses no protein precipitant was used other than the initial addition of dilute HCl.

A volume of 150 ml. rumen contents was withdrawn through the fistula of each of the three sheep fed on diets of hay, dried grass and casein respectively. It was filtered through muslin to remove the coarser plant particles and a half volume of 0·03 N-HCl added. The mixture was centrifuged at 18,000 *g* for 30 min and the supernatant liquid brought to pH 9 with 2N-KOH and placed in a vacuum desiccator over concentrated H_2SO_4. After 48 h the total volume was greatly reduced and over 95% of the ammonia had been removed. The syrupy liquid was made up to 15 ml. and the solid particles were removed by centrifuging at 12,000 *g* for 10 min. The material was placed in an electrolytic desalting apparatus (Shandon Scientific Co., London, S.W. 7) until the current fell to 0·05 amp. It was then again placed in a vacuum desiccator and dried over conc. H_2SO_4. The solid was washed out with small volumes of distilled water, made up to 1 ml. and filtered. The results of the examination of this concentrate by paper chromatography and the Van-Slyke amino-N method are given in Table 1.

Table 1. *The amino-acids of rumen contents of sheep*

(The results are given as the concentration of amino-N in the initial sample of rumen contents. Sample 1 was taken before feeding and sample 2 was taken 3 h after feeding)

Diet	Sheep no.	Sample	Amino-N (mg/100 ml.)	Number of spots observed by paper chromatography using: Butanol 50, water 40, acetic acid 10*	Phenol/ water†	Collidine/ water‡	Provisional identification
Hay and casein	8	1	0·34	8	5	4	Glycine, alanine, valine, methionine, glutamic acid, proline, leucine, serine, histidine
		2	1·40	9	5	4	
Hay and dried grass	12	1	0·28	6	5	3	Glycine, alanine, valine, methionine, lysine
		2	0·93	6	4	4	
Hay alone	24	1	0·12	4	2	2	Glycine, alanine, valine, arginine, α-aminobutyric acid
		2	0·72	3	2	1	

* See also Partridge & Westall (1948).
† NH_3, N_2, KCN (see also Block, 1950).
‡ See also Dent (1948).

Samples were taken and examined in this way from the three sheep immediately before and 3 h after feeding. Analyses were also made of samples of rumen contents from these sheep at the same times without any preliminary concentration. The material was desalted and the ammonia removed before examination. Using samples that had not been concentrated it was not possible to demonstrate conclusively the presence of amino-acid either by paper chromatography or by N released on nitrous-acid treatment though the results appeared to indicate their presence. When the material had been concentrated approximately a hundredfold it was clear that amino-acids were present in significant amounts. A comparison of the results from the three

sheep shows that the concentration of amino-acid was greatest in the sheep receiving casein (Table 1). The presence of amino-acids was definitely established, and by the chromatographic procedures at least five amino-acid spots were observed in most samples. The main spots were provisionally identified as glycine, alanine, valine, glutamic acid, methionine and proline. Using the Van Slyke amino-N procedure it was found that the concentration of amino-acids was within the range of 0·1–1·4 mg amino-N/100 ml. rumen contents. The concentration was higher after the sheep had fed.

Breakdown of amino-acids in the rumen

The present work was initially designed to determine the products of the breakdown of individual amino-acids and to examine the organisms responsible for such reactions. Preliminary experiments showed that individual amino-acids were not rapidly attacked in vitro by washed suspensions of rumen bacteria. It had, however, been shown (El Shazly, 1952*a*, *b*) that mixed amino-acids gave rise to ammonia and volatile fatty acids in the presence of rumen bacteria both in vivo and in vitro. Consequently, it was decided to determine whether amino-acids were attacked individually in vivo.

The three sheep were dosed with the following amino-acids: DL-alanine, L-glutamic acid, L-methionine and glycine; casein and casein hydrolysate were also administered. The results are presented in Fig. 1. In each sheep the values are given for the ammonia concentrations in the rumen after dosing with casein or amino-acids and also for the same periods when the sheep was not fed or dosed. The extent of ammonia production is taken as an index of the rate of breakdown of the amino-acids. There was a significant production of ammonia in all instances though the rate and extent varied. Ammonia production was most rapid in the casein-fed sheep and least rapid in the hay-fed sheep. Because of the rapid attack upon amino-acids in vivo the washed suspension of rumen bacteria was examined with a view to developing conditions under which amino-acids were more rapidly attacked.

Deamination by washed suspensions of rumen bacteria

Preliminary experiments were carried out in Warburg manometer flasks in a nitrogen atmosphere using washed suspensions of rumen micro-organisms. After a period of equilibration the individual amino-acids were added from a side-bulb and the mixture was incubated for 2 h at 37°. Ammonia was estimated at the end of this period after sulphuric acid had been added from a second side-bulb to stop the reaction. The results are presented in Table 2. It was considered that significant deamination occurred when the ammonia released in the presence of the amino-acid was at least double that in the control. In most instances the extent of ammonia production was very small. Significant deamination occurred only in the presence of L-aspartic acid and L-cysteine, although a small production of ammonia occurred in the presence of L-alanine, L-glutamic acid, L-serine and L-threonine. With all the other amino-acids tested the difference between the ammonia concentration in their presence and in the control was very slight. Total gas output was also measured and expressed as carbon dioxide; the only significant quantity produced was in the presence of L-aspartic acid and L-glutamic acid.

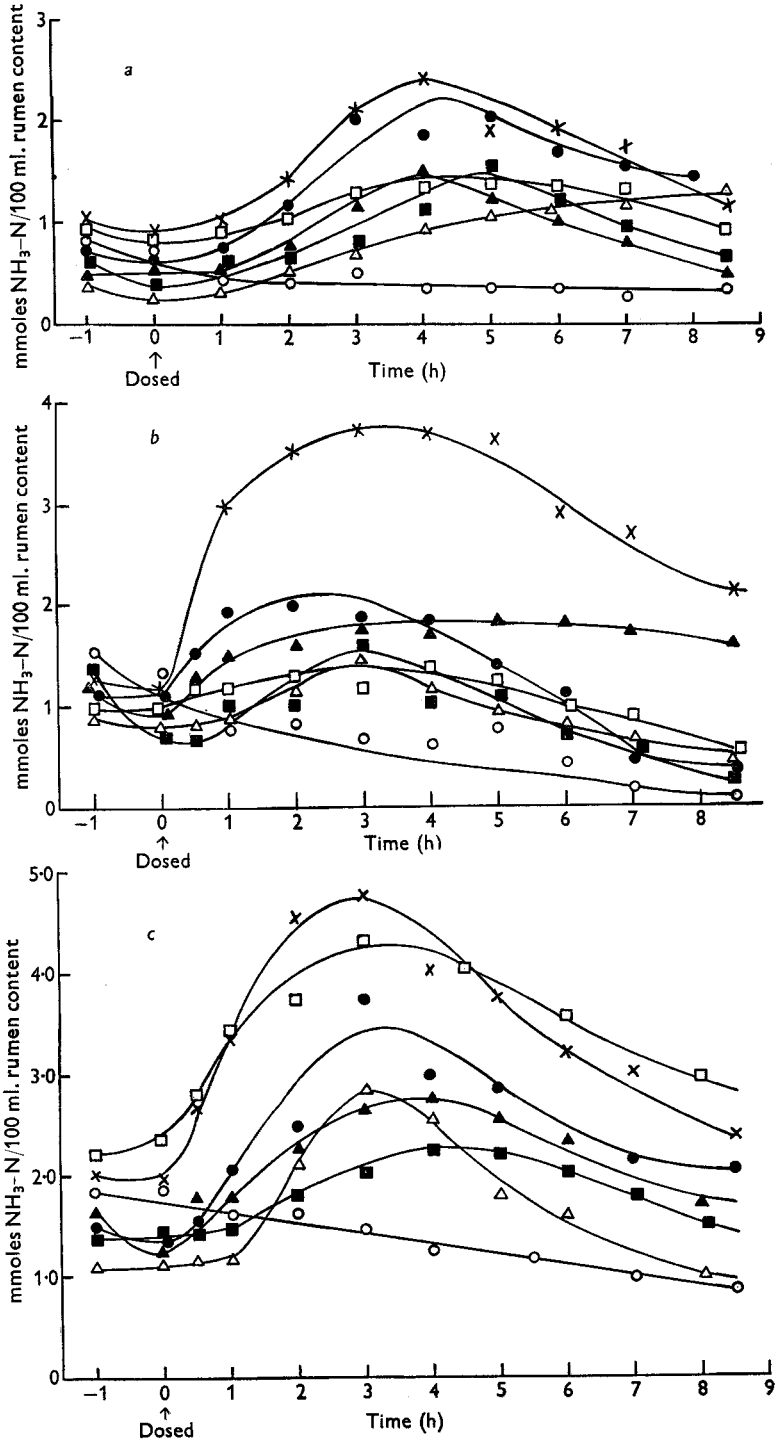

Fig. 1. Ammonia concentration in the rumen liquor of (*a*) sheep no. 24, fed on hay, (*b*) sheep no. 12, fed on hay and dried grass, and (*c*) sheep no. 8, fed on hay and casein, following a dose of amino-acid or protein approximately equivalent to 100 mg amino-N/kg live weight. No food was offered on the days of experiment. ○, no dose; ●, casein; △, DL-methionine; ×, casein hydrolysate; ■, glycine; □, DL-alanine; ▲, DL-glutamic acid.

In view of the small amounts of ammonia produced attempts were made to increase the extent of deamination by introducing a series of modifications to the technique. A summary of the results is given in Table 3, expressed as the quantity of ammonia produced when the amino-acid (50 μmoles) was incubated for 2 h with the washed suspension. It must be pointed out that the blank values were consistently high. In order to reduce the ammonia content and the gas evolved by control suspensions it was necessary to wash the micro-organisms several times. By this procedure it was found invariably that the activity was considerably reduced. A procedure for washing the organisms twice was adopted.

Table 2. *Ammonia production from amino-acids with washed suspensions of rumen micro-organisms*

(Experiments carried out in manometer vessels containing 2 ml. suspension (0·5–0·7 mg total N/ml.) in 0·1 M-phosphate buffer (potassium salts) pH 6·5, which contained 0·02 % (w/v) $Na_2S.9H_2O$. One side-bulb contained 50 μmoles substrate in 0·5 ml. H_2O and the other 0·5 ml. 5N-H_2SO_4. Incubation for 2 h at 37° in N_2 atmosphere. The values given for amino-acids represent the total ammonia minus the control values)

Substrate	NH_3 (μmoles)	CO_2* (μmoles)	Substrate	NH_3 (μmoles)	CO_2* (μmoles)
Control	9·8	8·4	Control	13·3	8·8
L-Threonine	6·8	4·3	L-Aspartic acid	18·8	12·5
L-Cysteine	14·5	6·8	L-Tyrosine	0·2	0
L-Serine	7·9	4·3	L-Lysine	0	0
L-Valine	0·3	0	L-Phenylalanine	1·3	0·7
L-Leucine	0	0	L-Methionine	1·8	0·5
Control	11·1	7·7	Control	8·7	3·9
L-Alanine	7·8	4·6	L-Proline	0·1	0
L-Glutamic acid	9·3	10·5	L-Hydroxyproline	0	0·3
L-Histidine	3·3	0·8	L-Ornithine	0	0
L-Tryptophan	0·5	0	L-Arginine	0·8	0·8
Glycine	0	0	L-Isoleucine	0	0

* Values for total gas output are expressed as CO_2; changes in gas output not absorbed by KOH were found to be small.

The following variations in the techniques were introduced and results are given in Table 3.

Thicker suspension. In the usual preparation of the suspension a volume of buffer is used equal to half that of the sample of rumen contents initially withdrawn. This suspension then contains about 0·3–0·6 mg total N/ml. In Exp. A the volume of buffer used was reduced to one-fifth and the suspension contained 1·62 mg total N/ml. In the control experiment (Exp. O), the suspension contained 0·48 mg total N/ml. In Exp. A the extent of the reaction was greater than in the control but not significantly so in view of the larger number of organisms used.

Reaction in hydrogen atmosphere. The second series (Exp. B) was carried out in an atmosphere of hydrogen. The results show no difference from the control series when the incubation was in a nitrogen atmosphere (Exp. O).

More alkaline pH. Bacterial deaminases (Gale, 1940) are known to have pH optima on the alkaline side of neutrality. Since the preliminary experiment was carried out at pH 6·5, a further series (Exp. C) was tested in 0·1 M-borate buffer at pH 8·5. This procedure again did not result in a significantly different rate of deamination.

Stricter anaerobiosis. A series of experiments was carried out with more attention given to the maintenance of anaerobiosis. All the reagents were maintained oxygen-free by boiling and cooling them rapidly before use. They were then gassed with nitrogen which contained not more than five p.p.m. oxygen. Yellow phosphorus was placed in the centre-wells of the manometer vessels. Though there appeared to be a slightly greater degree of deamination in some flasks (Exp. D) the results were not significantly different.

Table 3. *Attempts to increase ammonia production from amino-acids incubated with washed suspensions of rumen micro-organisms*

(Experiments carried out in manometer vessels under conditions given in Table 2, but modified in A to K as described in the text, p. 220. Incubation lasted for 2 h at 37°; all the results, given in μmoles, have been corrected for the control values)

	Experiment										
Substrate	O	A	B	C	D	E	F	G	H	I	K
Control	8·6	23·6	7·3	7·9	8·4	7·8	8·4	10·6	14·6	8·8	15·5
L-Alanine	6·3	8·4	6·3	4·6	8·3	4·7	5·6	8·9	10·8	27·8*	15·5
L-Aspartic acid	18·9	25·3	15·6	15·9	22·6	18·9	16·3	21·6	33·5	28·4†	38·8
L-Cysteine	10·5	18·6	11·5	11·0	14·3	12·6	13·3	12·9	15·6	21·8‡	15·6
L-Glutamic acid	4·8	4·5	7·4	7·2	6·9	5·4	8·6	10·5	14·8	8·8†	12·3
Glycine	0·2	0·8	0·1	0	0·3	0	0·5	1·3	2·3	12·6‡	4·6
L-Histidine	2·8	3·9	3·3	3·3	3·6	3·9	8·8	7·6	12·5	6·6†	5·8
L-Lysine	0·3	0·5	0	0	0	0·2	0·2	1·3	1·3	12·6‡	4·7
L-Methionine	1·1	0·8	0	0	0	1·1	0·3	0·3	0	2·8*	5·5
L-Phenylalanine	0·3	1·3	0·1	0·3	0·8	1·0	0·8	2·3	0·8	2·6*	4·4
L-Proline	0	0·1	0·1	0·5	1·8	0	1·0	1·6	0	25·5‡	4·4
L-Leucine	0·1	0·5	0	1·0	1·8	1·1	0·8	0	0	2·2†	1·6
L-Valine	0	0	0	1·5	3·6	0·8	1·1	2·6	2·6	4·5*	3·8

* Also containing 50 μmoles proline.
† Also containing 50 μmoles glycine.
‡ Also containing 50 μmoles alanine.

Other redox agents. An attempt was made to use alternative agents for the maintenance of a suitable redox potential. Sodium sulphide (0·2%, w/v) was replaced at the same concentration by thioglycollic acid (Exp. E), L-cysteine (Exp. F) and glutathione (Exp. G). Deamination was slightly more rapid in the presence of glutathione than without it, though the control ammonia level was also higher.

Seitz-filtered rumen contents. The addition to the phosphate buffer of an equal volume of Seitz-filtered rumen contents (Exp. H) gave with some amino-acids an increased rate of ammonia production. Before the Seitz-filtered rumen contents were added to the buffer, ammonia was removed in a vacuum desiccator over concentrated sulphuric acid at pH 9·0. The pH was then adjusted to 6·5 before the rumen filtrate was added to the buffer.

Pairs of amino-acids. In view of the results of El-Shazly (1952*b*) showing that a Stickland type of reaction could be brought about by washed suspensions, a series of pairs of amino-acids was tested. Only with L-alanine and L-proline together was the extent of deamination significantly greater than would be expected in the presence of the amino-acids individually (Exp. I).

Combined modifications. None of the variations introduced resulted in a marked

increase in deamination. Finally an experiment was carried out (Exp. K) in which the modifications resulting in any slight increase in deamination were tried together. A suspension was used containing 1·06 mg total N/ml. The suspending medium was composed of equal volumes of an ammonia-free Seitz-filtered rumen fluid and 0·1 M-phosphate buffer pH 7·0 containing 0·02% (w/v) glutathione. Yellow phosphorus was placed in the centre-wells of the manometer vessels and special care was taken in the maintenance of anaerobiosis. With several of the amino-acids there was a slight increase in ammonia formation under these conditions.

Products in amino-acid deamination

In view of the slightly increased rate of deamination recorded in Exp. K (Table 3) the products were determined during the breakdown of a group of amino-acids under those conditions, i.e. using the combined modifications that had to any extent increased ammonia production. The amino-acids used were L-aspartic acid, L-threonine, L-serine, L-alanine, L-cysteine and L-glutamic acid. These experiments were carried out in the way described by Elsden & Lewis (1953). The carbon dioxide evolved was

Table 4. *Products in the fermentation of amino-acids by washed suspensions of rumen contents*

(Experiments in manometer vessels containing 2 ml. suspension, 0·68 mg total N/ml. in 0·1 M-phosphate buffer pH 7·0 and Seitz-filtered rumen contents. One side-bulb contained 0·2 ml. CO_2-free 2N-NaOH and filter-paper, the other 0·4 ml. 4N-H_2SO_4. Substrates added from a Keilin dangling tube. Incubation lasted for 2 h at 37° in N_2 atmosphere. All results have been corrected for the control values)

		μmoles formed			
Substrate	μmoles added	NH_3	CO_2	H_2	Volatile fatty acids
Control	—	15·8	18·3	3·8	16·1
L-Aspartic acid	30·4	25·3	21·7	5·6	23·1
L-Threonine	26·8	8·4	5·6	0·2	6·6
L-Serine	33·0	16·2	11·1	2·8	16·0
L-Alanine	35·4	16·9	10·5	6·2	16·9
L-Glutamic acid	38·1	12·2	11·4	1·8	12·0
L-Cysteine	30·0	17·4	13·8	3·8	9·4

estimated and the gas not absorbed by KOH was measured and presumed to be hydrogen. Ammonia and volatile fatty acids were determined. The results (Table 4) show that with all amino-acids there was some production of ammonia, carbon dioxide and volatile fatty acids. With the exception of aspartic acid the quantity of fatty acid produced was not sufficient to allow an accurate measure of the individual fatty acids by the James & Martin (1952) procedure. By the use of a paper-chromatographic method (Elsden & Lewis, 1953) it was shown that acetic acid was probably formed from serine, alanine, glutamic acid and cysteine, and that an increase in propionic acid over that found in the control occurred in the presence of aspartic acid and threonine. It was not possible to demonstrate the formation of lactate or of ketone bodies. Little hydrogen was released, except during the incubation with L-aspartic acid and L-alanine.

Effect of diet

In view of the work of Annison, Chalmers, Marshall & Synge (1954) it was likely that the nature of the diet of the experimental sheep would have a considerable effect on the rate of deamination by the washed suspensions. To test this hypothesis the three sheep fed on the different diets described were used and the washed suspensions of rumen micro-organisms were prepared in the usual way. The suspensions were incubated with single amino-acids and the extent of deamination after 60 min, assessed in terms of ammonia, was determined. The results are expressed as μl. NH_3/mg total N/h (Table 5). This presentation corrects for any differences that might arise because of varied strengths of the suspensions. As expected from the in vivo studies, deamination was more rapid by the washed suspensions prepared from the sheep fed on the casein supplement than by those obtained from the other two sheep.

Table 5. *Rate of ammonia production from individual amino-acids by washed suspensions from sheep fed on different diets*

(Experiments in manometer vessels containing 2 ml. suspension; one side-bulb contained approx. 50 μmoles amino-acid and the other 0·5 ml. 4N-H_2SO_4. Incubation lasted for 60 min at 37° in N_2 atmosphere. Results are expressed as μl. NH_3/mg total N/h and are corrected for the control values)

	Sheep no.		
	24	12	8
Substrate	(Hay diet, suspension 0·65 mg total N/ml.)	(Dried-grass diet, suspension 0·73 mg total N/ml.)	(Casein diet, suspension 0·84 mg total N/ml.)
Control	59	87	105
L-Aspartic acid	104	165	215
L-Cysteine	63	109	121
L-Alanine	14	49	58
L-Serine	36	38	71
L-Threonine	29	31	65
L-Valine	0	10	15
Glycine	0	0	9
L-Methionine	5	10	12
L-Arginine	0	0	3

Fermentation of aspartic acid by washed suspensions

Since the deamination of aspartic acid by washed suspensions is comparatively rapid, it was possible to examine further the products of the reaction and the factors that affect the deamination. Aspartic acid was incubated with a washed suspension in nitrogen atmosphere. The evolution of hydrogen and carbon-dioxide was estimated by the method of Elsden & Lewis (1953), together with the ammonia and volatile fatty-acid production. No ketone bodies or lactic acid were formed. The results of the analyses (Table 6) show that there was a large evolution of carbon dioxide with a small quantity of hydrogen and approximately equimolar amounts of volatile fatty acid and ammonia. The main component of the volatile fatty-acid fraction was propionic acid with smaller amounts of acetic and *n*-butyric acids. These are the main

components of the volatile fatty acids in the rumen, so the products of the breakdown of aspartic acid may contribute to the fatty acids normally found in the rumen.

It was observed in some of the earlier experiments that increasing the amount of aspartic acid did not appreciably increase the quantity of ammonia released. Also, since amino-acids are normally present in the rumen in low concentrations, it was

Table 6. *Fermentation of aspartic acid by washed suspensions of rumen contents*

(Manometer vessels contained 2 ml. suspension, 0·65 mg total N/ml., and 0·2 ml. CO_2-free 2 N-NaOH in one side-bulb, with 0·4 ml. 4 N-H_2SO_4 in the other. Substrate added from Keilin dangling tube (30·4 μmoles aspartic acid) and incubation for 2 h in N_2 atmosphere. All results are expressed as μmoles and are corrected for the control values)

Product	Control value	Corrected experimental value
Ammonia	13·8	22·6
Carbon dioxide	10·1	21·8
Hydrogen	2·9	2·7
Volatile fatty acids	22·6	23·2
Formic acid	1·1	—
Acetic acid	13·6	3·4
Propionic acid	6·2	18·4
Butyric acids	1·2	1·2
Valeric acids	0·5	0·2

Table 7. *Effect of concentration of substrate on degree of deamination of aspartic acid*

(Manometer vessels contained 2 ml. suspension, 0·55 mg. total N/ml., and substrate added in 1 ml. volume from side-bulb. Incubation lasted for 60 min at 37° in N_2 atmosphere. Results have been corrected for the control values)

Substrate added (μmoles)	CO_2 (μmoles)	NH_3 (μmoles)	Substrate deaminated (%)
Control	12·2	11·4	—
6·4	6·2	6·5	100
12·8	10·3	9·9	78
32·0	29·5	21·8	68
64·0	44·1	38·4	60
128	34·7	41·2	32
192	19·6	16·8	8

desirable to determine the rate of deamination of aspartic acid at various concentrations. The results are presented in Table 7 and show that up to about 130 μmoles aspartic acid there was a slow increase in the amount of ammonia released under the conditions described. However, under these conditions the percentage attacked fell off over the whole range of 6–190 μmoles as the substrate concentration was increased. When the quantity of substrate added to 2 ml. of the suspension was increased to 192 μmoles there was an actual decrease in the total amount of ammonia released. It may be tentatively suggested that it was due to a substrate-inhibition effect. The results, however, show clearly that with low concentrations of aspartic acid there was rapid deamination. Such an ability may be effective in maintaining the comparatively low concentration of amino-acids in rumen contents.

The effect of changes in the hydrogen-ion concentration of the medium on the rate of deamination of aspartic acid by the washed suspensions was examined. The incubations were carried out in an atmosphere of nitrogen for 40 min, and at this point the reaction was stopped and the ammonia production estimated. In this experiment the organisms were washed with, and suspended in, 0·9% (w/v) sodium-chloride solution containing 0·02% (w/v) thioglycollic acid and the pH was then adjusted to 7·0. The manometer vessels contained 1 ml. of this suspension and 1 ml. 0·2M-phosphate buffer (for the range of pH 6–8) or 0·2M-borate buffer (above pH 8). The results (Fig. 2) are expressed in the form of the percentage of the maximum rate

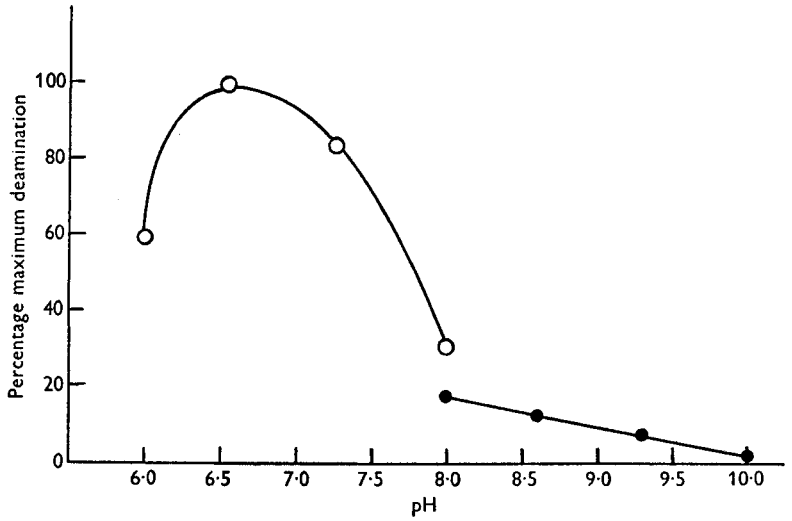

Fig. 2. Effect of changes in the hydrogen-ion concentration on the rate of ammonia production from aspartic acid. Each manometer vessel contained 1 ml. washed suspension (0·51 mg total N/ml.) and 1 ml. 0·2M buffer. Phosphate buffer (○) for pH 6–8 and borate buffer (●) for pH 8–10. Other details in text.

of ammonia production and show clearly that the optimum pH was about 6·5, which is in the middle of the normal range of values for the rumen of the sheep and agrees well with other results obtained when using washed suspensions prepared in this way (Lewis, 1951; Sijpesteijn & Elsden, 1952; Lewis, 1954).

Fermentation of pairs or groups of amino-acids

The work of El-Shazly (1952*b*) suggested that the attack upon amino-acids in the rumen of the sheep follows the pattern of the Stickland reaction (see Nisman, 1954). He demonstrated that alanine and proline underwent a Stickland type of reaction and found that δ-aminovaleric acid was produced. An attempt has been made to extend this observation by using pairs of other recognized hydrogen donors and acceptors. However, in no other instance was it possible to show that the ammonia produced in the presence of a pair of amino-acids was significantly greater than when each was incubated individually with the suspension. In view of this an experiment was carried

out in which the washed suspension was incubated with an acid hydrolysate of casein (El-Shazly, 1952*b*) and also with an artificial mixture of amino-acids similar in composition to a casein hydrolysate. Both these mixtures were rapidly deaminated. Therefore it was possible that a smaller group of amino-acids might form a basic unit required for rapid deamination. A series of experiments was thus designed in which a variety of mixtures of amino-acids was incubated with washed suspensions.

Table 8. *Ammonia production from mixtures of amino-acids*

(Vessels contained 2 ml. suspension, 0·83 mg (A) and 0·91 mg (B) total N/ml. and substrates in side-bulb. In series A, 10 μmoles of each amino-acid in 0·1 ml. H_2O and in series B a total of 50 μmoles substrate were added to each incubation which lasted for 2 h at 37° in N_2 atmosphere. Results are corrected for the control values. L-Amino-acids were used throughout. Ala, alanine; Arg, arginine; Asp, aspartic acid; Cy–SH, cysteine; Glu, glutamic acid; Gly, glycine; His, histidine; Leu, leucine; Met, methionine; Pro, proline; Phe, phenylalanine; Ser, serine; Thr, threonine; Try, tryptophan; Tyr, tyrosine; Val, valine; CH, casein hydrolysate)

Series A			Series B		
No. of amino-acids added	Amino-acids	μmoles NH_3	No. of amino-acids added	Amino-acids	μmoles NH_3
0	Control	13·1	0	Control	13·8
1	Alanine	9·7	1	Alanine	10·5
1	Arginine	6·3	1	Arginine	7·5
1	Aspartic acid	9·3	1	Aspartic acid	19·4
1	Cysteine	9·5	1	Cysteine	10·5
1	Glutamic acid	5·3	1	Glutamic acid	6·3
1	Glycine	3·2	1	Glycine	1·1
1	Histidine	5·2	1	Histidine	2·5
1	Leucine	2·8	1	Leucine	2·2
1	Methionine	3·8	1	Methionine	3·6
1	Proline	3·3	1	Proline	0·3
1	Phenylalanine	3·7	1	Phenylalanine	3·6
1	Serine	6·5	1	Serine	7·9
1	Threonine	6·2	1	Threonine	10·6
1	Tryptophan	2·6	1	Tryptophan	4·4
1	Tyrosine	2·9	1	Tyrosine	3·8
1	Valine	1·1	1	Valine	4·6
—	CH	8·2	—	CH	29·6
16	Mixture of above sixteen (A)	41·8	16	Mixture of above sixteen (A)	32·3
14	A less Try and Tyr (B)	43·4	14	A less Asp and Cy–SH (L)	30·9
12	B less Asp and Cy–SH (C)	29·2	12	L less Glu and His (M)	27·3
10	C less Glu and His (D)	16·7	10	M less Ser and Thr (N)	25·3
8	D less Ser and Thr	15·0	8	N less Tyr and Try	26·1
4	Asp with Cy–SH and Glu and His (E)	24·3	4	Asp with Cy–SH and Glu and His (E)	22·9
6	E with Ser and Thr (F)	28·0	6	E with Ser and Thr (F)	24·8
8	F with Try and Tyr	29·6	8	F with Gly and Pro	25·5
4	Glu with His and Ser and Thr (G)	13·9	5	Ala with Glu and Leu and Pro and Val (P)	25·3
7	G with Ala and Gly and Pro (H)	20·2	8	P with Arg and Met and Phe	27·5
11	H with Asp and Cy–SH and Leu and Val (I)	35·2	6	Ala with Asp and Gly and Glu and Pro and Val	21·8
9	I less Asp and Cy-SH (J)	20·2	4	Ala with Gly and Leu and Pro	18·4
7	J less Glu and His (K)	15·2	4	Gly with His and Ser and Thr	20·6
5	K less Ser and Thr	13·2			

Blanks were arranged in the absence of substrate, and a series of controls was set up in the presence of each amino-acid individually. Two types of experiment were carried out and a typical series of results is given in Table 8. In one series the total quantity of amino-acid incubated with the suspension was the same irrespective of the number of amino-acids present, whereas in the other series the amount of each amino-acid added whether alone or in a mixture was the same.

The quantity of ammonia produced in these incubations gives a measure of the extent of deamination of the added amino-acids, for it was confirmed in some preliminary experiments that disappearance of amino-acids paralleled ammonia production. This disappearance of amino-acids was tested by the Van Slyke amino-N method and by a visual examination of paper chromatograms. During the incubation of a mixture of amino-acids including aspartic acid it was observed that within 2 h aspartic acid almost completely disappeared. The quantity of the other amino-acids in the mixture was also slowly reduced.

It is clear from the results obtained (Table 8) that there was no definite group or number of amino-acids necessary to give rapid deamination. The extent of the ammonia production with a mixture of sixteen amino-acids was comparable to that with casein hydrolysate in series B. The material used in Series A was not a complete hydrolysate. As the number of amino-acids in the mixture was reduced the quantity of ammonia released slowly fell. The larger decreases were found when aspartic acid or one of the group of amino-acids shown to be significantly attacked individually was omitted. Furthermore, a mixture of amino-acids shown individually not to be significantly deaminated was attacked under these conditions. It is clear therefore that a rapid deamination of amino-acids by washed suspensions of rumen micro-organisms, with a few exceptions, requires the presence of a fairly large mixture of amino-acids. The products under these conditions have not been estimated and no explanation is offered for the mechanism of the process.

DISCUSSION

Following the work of McDonald (1948, 1952) in demonstrating the importance of ammonia production in the rumen, Chalmers, Cuthbertson & Synge (1954) were able to show convincingly that the extent of ammonia production in the rumen from a protein foodstuff is of considerable significance in the nitrogen metabolism of the ruminant. Their results indicated that the value of protein to the ruminant varied inversely with the rate at which it was attacked in the rumen. From the more recent work in this field it may be suggested that ammonia production in the rumen is governed by many factors, among them being the solubility of the protein ingested, the state of division of the protein particles, the degree of denaturation or the extent of processing in the preparation of the foodstuff and the proportion of carbohydrate material present. Since it is probable that the immediate source of ammonia in the rumen is the amino-acid mixture produced from protein, it is necessary to understand the conditions under which ammonia is released from these substances. The present work was designed for such a purpose.

The experiments in vivo using the sheep fitted with permanent rumen fistulas have shown that the amino-acids tested are attacked individually by rumen micro-organisms, and that amino-acids are found in rumen contents in comparatively low concentrations. These results strengthen the probability that amino-acids are intermediates in the breakdown of protein in the rumen, but do not prove it. The concentration of amino-acids in rumen contents was within the range of 0·1–1·4 mg amino-N/100 ml., and the main amino-acids present were identified. It is noteworthy that amongst the most prominent were glycine, alanine, valine and leucine. The lower limit for the detection of individual amino-acids in rumen contents by the methods used is of the order of 0·05 mg amino-N/100 ml. It is possible that other amino-acids occur in rumen contents, but that the concentration is too low for identification by the methods employed.

El-Shazly (1952*b*) has shown that casein hydrolysate is fermented in the rumen of the sheep and also by washed suspensions of rumen contents. This finding has been confirmed and it has also been shown that a synthetic mixture of amino-acids is broken down in the same way by washed suspensions. However, when the amino-acids were tested singly in vitro only L-aspartic acid was rapidly deaminated, and L-alanine, L-glutamic acid, L-serine, L-threonine and L-cysteine more slowly. While this work was in progress Sirotnak, Doetsch, Brown & Shaw (1953) presented results to indicate that some individual amino-acids are attacked by washed rumen organisms. Their findings agree qualitatively with those reported here, but it is difficult to assess the significance of some of their results. Some of the incubations were continued for very long times without examination of the micro-organisms. Also, little indication is given of the diet of the sheep or of the control values. However, in view of the expected high blanks, their results show a remarkably good agreement between ammonia, carbon-dioxide and volatile fatty-acid production.

Since the rate of attack upon single amino-acids in vivo was considerably more rapid than when washed suspensions were used, it seemed probable that the method of preparation of the suspension in some way rendered inactive the enzymes responsible for the deamination. Increases in the activity were obtained by using greater care in the maintenance of anaerobiosis, by the addition to the medium of a proportion of ammonia-free Seitz-filtered rumen contents and by the use of a thicker suspension with phosphate buffer at pH 7 containing 0·02% (w/v) glutathione. However, ammonia production from most of the amino-acids when tested singly was still comparatively slow so that the conditions may still have differed significantly from those present in the rumen.

Consideration was given to the possibility of a Stickland type of reaction but no increased rate of ammonia production was obtained except with alanine and proline. However, by increasing the numbers of amino-acids incubated with the suspensions it was possible to obtain a greater ammonia production. This finding is difficult to explain; it may be that certain co-factors in the rumen are lost and replaced by the mixed amino-acids; that an inhibitory material is produced which in the absence of the variety of substrates is not further metabolized; that a complex process involving growth or peptide formation is needed, or that some influence is exerted on the redox potential.

In addition to deamination of a mixture of amino-acids by the washed suspensions, it has also been shown in vitro that aspartic acid is fermented, that certain other amino-acids are slowly attacked and that alanine and proline together (El-Shazly, 1952*b*) give rise to ammonia. The attack upon aspartic acid followed a typical pattern of bacterial fermentation in that ammonia, carbon dioxide, volatile fatty acids and hydrogen were formed. The pH optimum for the ammonia production was around 6·5. When the substrate concentration was increased from low levels, less than the expected increases in ammonia and carbon-dioxide production were observed. Further attention must be given to explain this finding. In the same way it was shown that during the slow attack upon other amino-acids the products were ammonia, carbon dioxide, volatile fatty acids and hydrogen. All these products, with the exception of hydrogen, are normally found in the rumen.

SUMMARY

1. A preliminary study has been made of the amino-acid content of the rumen liquid of sheep fed on hay alone and on hay supplemented with casein or with dried grass.

2. The amino-nitrogen varied from 0·1 to 1·4 mg/100 ml. rumen liquid. It was lowest in the sheep receiving hay only and highest in the sheep that received hay supplemented with casein. On all three diets it was higher 3 h after feeding than immediately before feeding.

3. A study has also been made of the production of ammonia from amino-acids both in the rumen and also by washed suspensions of rumen bacteria in vitro.

4. Individual amino-acids placed in the rumen gave rise to ammonia.

5. When individual amino-acids were incubated with washed suspensions of rumen bacteria, aspartic acid appeared to give the greatest production of ammonia. At the same time volatile fatty acids, CO_2 and hydrogen were formed. Some other individual amino-acids also gave ammonia under the same conditions, but much more slowly than aspartic acid. Mixtures of several amino-acids were rapidly deaminated.

6. Some of the factors likely to influence ammonia formation were studied. The optimum pH for the process was found to be about 6·5, a value typical for rumen contents in the sheep.

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An Investigation of the Daily Intakes of Food of Individual Boys at a Boarding School in Uganda

BY RUTH SCHWARTZ AND R. F. A. DEAN

Medical Research Council Group for Research in Infantile Malnutrition, Mulago Hospital, Kampala, Uganda

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The original purpose of the investigation was to measure the amount of food eaten by boys in the school, which was a boarding school, at a time when an unpopular diet had to be given because of high prices and shortages of better-liked foods. It was the best the school authorities could devise, but they had doubts of its value, and whether the boys were eating enough. The food eaten daily by thirty boys was therefore measured for 1 week. In the following term it proved possible to re-introduce better-liked foods, and the intakes of the thirty boys were measured again. The school food was not very ample so, to test its adequacy, nineteen of the boys were allowed to eat as much of it as they wanted for a further week during which their intakes were measured for the third time.

EXPERIMENTAL

Background of the investigation

Details of the school. The school is in the province of Buganda about 11 miles from Kampala. A large proportion of its pupils are children of chiefs or other prominent and prosperous members of the community; 85% are Baganda, an autochthonous Bantu tribe. The school fees are high by local standards but they defray only a fraction of the cost of the school; the rest is paid by a Uganda Government grant.