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Nitrogen Metabolism in the Sheep

PROTEIN DIGESTION IN THE RUMEN

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The economic importance of ruminants has ensured the continuous investigation of their nutritional requirements, which, it is now clearly recognized, are profoundly influenced by the presence of the rumen. Ingested food is subjected to microbial attack in the rumen of these animals, a process which is essential for the utilization of the roughage which normally forms a substantial part of their diet. Fermentation in the rumen, however, results in the degradation of some of the dietary protein to volatile fatty acids and ammonia, a process which in some circumstances is wasteful when the overall economy of the animal is considered.

Knowledge of protein digestion in the rumen has increased rapidly during the past decade, and an excellent review of the subject has recently appeared (Chalmers & Synge, 1954*a*). The proteolytic activity of rumen contents was recognized by Sym (1938), and Pearson & Smith (1943) obtained evidence for the proteolysis of casein and gelatin incubated with rumen contents. Hoflund, Quin & Clark (1948) found that proteins in fibre form dissolved slowly in the rumen, but the only quantitative measurements of protein breakdown in the sheep rumen are those made by McDonald (1954), who demonstrated that approximately 40 % of ingested zein was degraded. Warner (1955) has investigated the proteolytic activity of washed suspensions of rumen microorganisms, and has studied the degradation of a number of proteins incubated in an 'artificial rumen' similar to that used by Louw, Williams & Maynard (1949). In the presence of toluene, which suppressed the activity of the microbial deaminases, protein was degraded largely to amino acids.

The significance of ammonia as an end product of protein digestion in the sheep rumen was first clearly recognized by McDonald (1948, 1952), who demonstrated enhanced ammonia concentrations in the rumen after the ingestion of certain proteins. Subsequent investigations have confirmed and extended these observations (El-Shazly, 1952*a*; Chalmers, Cuthbertson & Synge, 1954; Annison, Chalmers, Marshall & Synge, 1954). Although proteins are almost certainly converted into free amino acids before degradation to ammonia in the rumen, the concentration of free amino acids is usually low, presumably because of their rapid uptake or degradation (McDonald, 1952). Several workers have investigated the degradation of amino acids by rumen organisms *in vitro*, and to simplify the system to allow the accurate analysis of reaction products the washed-suspension method of Sijpesteijn & Elsden (1952) has usually been employed. Thus El-Shazly (1952*b*), who studied the degradation of casein hydrolysate incubated with washed suspension of rumen organisms, found that ammonia and volatile fatty acids were the principal end products, and the formation of δ-aminovaleric acid from alanine and proline by a Stickland-type reaction was also demonstrated. Sirotnak, Doetsch, Brown & Shaw (1953) and Lewis (1955) have examined the behaviour of individual L-amino acids by the washed-suspension method, but only aspartic and glutamic acids, serine, cystine and arginine were found to be attacked. The dissimilation reactions of aspartic acid incubated with bovine-rumen bacteria were subsequently investigated by Sirotnak, Doetsch, Robinson & Shaw (1954).

In the present work the digestion of protein in the rumen of sheep fed on various diets has been investigated. The rumen ammonia and volatile fatty acid production in sheep fed on similar diets was

reported earlier (Annison *et al.* 1954; Annison, 1954). The degradation of proteins *in vitro* by washed suspensions of rumen bacteria has been studied, and the breakdown products have been examined in some detail. Free α-amino nitrogen was estimated by the specific ninhydrin–carbon dioxide method of Van Slyke, Dillon, MacFadyen & Hamilton (1941). The application of this method to sheep-rumen liquor indicated that appreciable amounts of free α-amino nitrogen (0·4–3·0 mg. of N/100 ml.) are always present, and the variations in concentration of this constituent under different dietary conditions have been investigated. During the course of this work Chalmers & Synge (1954*a*) reported concentrations of 0·4–4 mg. of α-amino nitrogen/100 ml. in sheep-rumen liquor, determined by a similar procedure. Investigation of the distribution of α-amino nitrogen between the micro-organisms and the liquid of rumen contents has shown that a considerable part of the free amino acids are associated with the micro-organisms. Much of this α-amino nitrogen is 'bound' to the micro-organisms in a manner similar to that typical of certain Gram-positive bacteria (Gale, 1953).

Young grasses contain appreciable quantities of free amino acids (Waite & Boyd, 1953), and it is likely that when ruminants graze on lush pastures the concentration of free α-amino nitrogen in the rumen becomes appreciably greater than that in the blood. Levels of α-amino nitrogen of the order 10–15 mg./100 ml. have been found in the rumen liquor of sheep during and immediately following the consumption of dried grass, and here the possibility arose that absorption of amino acids from the rumen might occur under these conditions. Experiments involving the analysis of portal blood after casein hydrolysate had been added to the rumen of sheep, however, suggested that amino acids are not absorbed from the rumen.

MATERIALS AND METHODS

Experimental animals. Clun Forest sheep with permanent rumen fistulae were employed. Two animals received special rations devised by Dr I. W. McDonald during another investigation. The basal diets of these sheep were similar, and consisted of 600 g. of straw (alkali-washed), 150 g. of starch, 100 g. of sucrose, 200 g. of molasses and 500 g. of water. The straw was steeped in 1·5 % NaOH for 24 hr., washed free from alkali and dried at 30–40°, a procedure which decreased the water-soluble N-containing materials to negligible amounts. The total N (Kjeldahl) of the treated straw was 0·2–0·3 %. In addition, the two sheep were fed on daily supplements of 100 g. of casein (sheep 1) and 100 g. of casein digest (sheep 2). The casein digest (N, 12·7 %; free α-amino N, 1·2 %) was obtained from Allen and Hanbury Ltd., Bethnal Green, London. An additional five sheep were given the following diets in the amounts shown (per day): hay (1 kg.), sheep 3; hay (1 kg.) and casein (150 g.), sheep 4; dried grass (600 g.), sheep 5; alkali-washed straw (300 g.) and flaked maize (600 g.), sheep 6; hay (300 g.) and groundnut meal (300 g.), sheep 7. The animals were trained to consume the ration within 1–2 hr.

Proteins used for in vitro *experiments.* The soluble proteins casein and bovine albumin and the partially soluble plant proteins arachin, zein, wheat gluten and 'α protein' (from soya bean) were used. These contained, respectively, 12·9, 14·2, 15·5, 14·2, 13·1 and 14·7 % of N. Casein (British Drug Houses Ltd.) was dissolved in 0·1 M phosphate buffer, pH 7·0, and dialysed at 1° against six changes of the same buffer. The solution (approximately 1 %) was then filtered through a Seitz pad and stored under sterile conditions. An aqueous solution (1 %) of crystalline bovine albumin (Armour Laboratories Ltd., U.S.A.) was prepared in a similar manner after dialysis against water. The plant proteins arachin (Imperial Chemical Industries Ltd.), zein, wheat gluten and 'α-protein' (the last three materials were obtained from Nutritional Biochemicals Ltd., U.S.A.) were suspended in water and dialysed against water at 1° to remove diffusible material, and then freeze-dried.

Analytical methods

Total N. This was estimated by the Kjeldahl method with the procedure of Hiller, Plazin & Van Slyke (1948) and a modification suggested by Dr I. W. McDonald (private communication). Mercury, which is used as a catalyst in this method, interferes with the steam-distillation of ammonia, and in the original method zinc dust is added to the distillation mixture to immobilize the mercury. This is a troublesome procedure if a Markham still is used, since the vigorous evolution of hydrogen which occurs during steam-distillation under alkaline conditions produces a fine spray of alkali which results in high blanks, and the zinc is not removed completely when the still is emptied. Dr McDonald has shown, however, that most of the mercury can be removed by adding the zinc dust to an aqueous solution (15 ml.) of the digest. The amalgamated zinc settles out and portions of the aqueous digest can then be steam-distilled in the normal manner after the addition of excess of NaOH. Control experiments showed excellent recoveries of NH_3 when digests were distilled after this procedure.

Ammonia. The general procedure of McDonald (1948) was used to determine NH_3 in rumen contents. In the *in vitro* experiments the NH_3 content of the incubated suspensions was determined without prior deproteinization.

Volatile fatty acids. The methods reported earlier (Annison, 1954) were used for the estimation of the total concentration of volatile fatty acids in rumen liquor, and where necessary complete analyses of these were made by the gas–liquid partition chromatographic method of James & Martin (1952). Analyses for volatile fatty acids were made on the incubation mixtures used in the *in vitro* experiments by similar methods.

Blood α-amino N. The procedure of Hamilton & Van Slyke (1943) was used. Blood urea was determined before analysis for α-amino N, and if higher than 40 mg./100 ml. the modification involving incubation of the deproteinized solution with ninhydrin before carrying out the reaction was employed. Estimations were made on whole blood and plasma, heparin being used as the anticoagulant.

Blood urea. This was determined as described by Conway (1947).

Paper chromatography of amino acids. Qualitative analyses of amino acids were made by the one-dimensional

techniques of Roland & Gross (1954) with *sec.*-butanol–3% NH_3 soln. (3:1, v/v) as solvent (descending), and Clayton & Strong (1954) with ethyl methyl ketone–propionic acid–water (75:25:30, by vol.) and the ascending method. The chromatograms were developed by spraying with ninhydrin (1% in butanol) and heating at 100° for 5 min.

Estimation of α-amino N *in rumen contents.* The ninhydrin–CO_2 method is specific for free α-amino N, but Hamilton & Van Slyke (1943) reported some interference by plasma protein. For this reason strained rumen liquor was deproteinized before analysis, with picric acid, which exerts no influence on the subsequent reaction (Hamilton & Van Slyke, 1943). Preliminary experiments showed that it was necessary to acidify strained rumen liquor (15 ml.) to pH 2·0–2·5 with 1 ml. of 2N-H_2SO_4 before deproteinization with 3 ml. of 1% picric acid. After centrifuging (15 min., 3000 *g*) 5 ml. portions of supernatant were examined for α-amino N, with the procedure and apparatus described by Hamilton & Van Slyke (1943). Analyses were made at pH 2·5 by adding 100 mg. of citrate buffer (Van Slyke *et al.* 1941) to each sample. When the reaction is carried out at pH 2·5 there is a possibility of errors due to hydrolysis of peptides or CO_2 formation from uronides. A comparison of the results obtained when several samples of deproteinized rumen contents were examined in parallel at pH 2·5 and 4·7 indicated greater CO_2 production at the higher pH. This is possibly due to CO_2 evolution from γ-aminobutyric acid or β-alanine under these conditions, and analyses throughout the investigation were therefore made at pH 2·5.

When control experiments were made comparing α-amino N levels in deproteinized and untreated samples of strained rumen liquor, the α-amino N content of the untreated material was found to be 50–100% higher than in the deproteinized rumen liquor, results which could not be accounted for by the interference of protein in the untreated samples. In addition, 10 ml. samples of strained rumen liquor were dialysed against 55 ml. of water at 1° for 16 hr., with continuous agitation of the cellophan sac. The diffusates (50 ml.) were concentrated *in vacuo* at 25–30° at pH 7·0, and examined for α-amino N. Values similar to those obtained when whole rumen liquor was examined were obtained. These results suggested that deproteinization caused considerable losses of α-amino N, or that much of the α-amino N of untreated rumen liquor is associated with the micro-organisms and is not liberated on deproteinization. Since considerable use was subsequently made of dialysis at 1–2°, the possibility of α-amino N liberation or uptake under these conditions was examined. Control experiments with rumen liquor showed, however, that at 1° the NH_3 and α-amino N levels remained unchanged after 24 hr.

The dialysis procedure described above was adopted for the estimation of ruminal α-amino N levels, since this method allowed the contents of diffusible peptide N to be determined on the same samples (see below), and in addition gave results which were only slightly lower than those obtained by examining the whole suspension.

Determination of diffusible peptide N. Strained rumen liquor (10 ml.) or 15 ml. of the incubation mixtures used in the *in vitro* experiments was dialysed in cellophan tubing at 1° against 55 ml. of water for 24 hr., with continuous agitation of the dialysis sac. The diffusate (50 ml.) was adjusted to pH 7·0, concentrated *in vacuo* at 30–40° and made up to 15 ml., and 3 ml. portions were hydrolysed in sealed ampoules with 3 ml. of 12N-HCl at 100° for 16 hr. The hydrolysates were taken to dryness *in vacuo* over conc. H_2SO_4 and KOH pellets, and the residues dissolved in water and examined for free α-amino N. Corrections were made for the free α-amino N content of the concentrated diffusates. 'Diffusible peptide N' therefore represents the α-amino N which is liberated on hydrolysis of the diffusible peptides.

Incubation experiments with washed suspensions. The suspensions were prepared by the general procedure of Sijpesteijn & Elsden (1952), all operations being carried out in a cold room at 1°. Rumen contents, strained through two layers of muslin, were lightly centrifuged to remove plant particles and protozoa, and then centrifuged at high speed (14000 *g*) for 30 min. The residue was resuspended to the original volume with 0·1M phosphate buffer, pH 7·0, prepared from M-NaH_2PO_4 and M-K_2HPO_4 with appropriate dilution, and containing 0·02% of Na_2S. This buffer was made O_2-free before use. The suspension was centrifuged at high speed (14000 *g*) for 20 min., and the washing repeated, when the residue was finally resuspended in buffer to half of the original volume. Suspensions were adjusted to the required concentration, measured as N content, by dilution and turbidimetric measurement in a photoelectric colorimeter (Unicam SP. 200, orange filter). It was necessary to prepare calibration curves of turbidity against N (Kjeldahl) content for the washed suspensions from each sheep used, since there was considerable variation in the N content of washed suspensions of bacteria of similar optical density from different sheep. Suspensions containing 0·5 or 1·0 mg. of bacterial N/ml. were usually employed.

The large double side-bulb flasks described by Lewis & Elsden (1955) were used for the incubation experiments. The washed suspension (15 ml.) was placed in the main bulb and the substrate in a side bulb. The flasks were gassed with O_2-free N_2 and incubated in a bath at 37° with constant shaking (120/min.). Immediately on removal from the bath the flasks were cooled in crushed ice to prevent further reaction, and made up to 25 ml. The bacterial suspensions were not acidified to stop further reaction, since this procedure would have caused protein precipitation, with some loss of free α-amino N. The final suspension was analysed for NH_3, volatile fatty acids, α-amino N and peptide N by the methods described.

RESULTS

Rumen α-amino and ammonia levels on various diets. The levels of α-amino N and ammonia, and in some cases peptide N, were determined by the dialysis procedure on the rumen liquor of sheep 1, 2, 3 and 6, which had been fed on the diets described earlier. From the results shown in Fig. 1, it can be seen that, in general, increases in ammonia concentration parallel those of amino N. Since the levels of both constituents increased rapidly during feeding, it was of interest to examine the free α-amino N and ammonia content of the foodstuffs. Samples (10 g.) of the hay and dried grass used in these experiments were extracted three times with water (50 ml.) at room temperature and the total N, α-amino N and ammonia N of the combined extracts determined. The respective values for hay were 16·1, 3·19 and 3·9 mg., and for dried grass 17·1, 6·15 and 2·3 mg. The ammonia N measured under these conditions is

probably largely derived from amides which break down to ammonia under the alkaline conditions used in the estimation of ammonia. The results indicate that the large increases in rumen α-amino N and ammonia during and immediately after feeding dried grass are largely due to the presence of free α-amino N and labile amide N in the foodstuffs. For this reason the results obtained with sheep 1 are of particular interest since the rations on which it was fed, casein and alkali-washed straw, contained negligible amounts of free α-amino N or amide N. The sheep consumed the ration within 1 hr., and there were rapid increases in the α-amino N and ammonia concentrations above the resting levels, followed by a sharp fall in the α-amino N concentration (Fig. 1, *A*). The diet of sheep 2 included 100 g. of casein digest (total N, 12·7 %; free α-amino N, 1·2 %) and the results (Fig. 1, *B*) indicate the rapid utilization or degradation of α-amino N in the rumen of this animal. Low ammonia concentrations in the rumen which occur when sheep are fed on flaked maize (Annison *et al.* 1954) are paralleled by low α-amino N levels (Fig. 1, *D*).

Influence of carbohydrate on rumen α-amino N, *peptide* N *and ammonia levels.* Concentrations of these constituents in the rumen of sheep 1 and 2 were followed under the usual feeding conditions (Fig. 1, *A*, *B*) and also when the starch and sucrose components were, for the day of the experiment only, omitted from the diets (Fig. 2). Omission of starch and sucrose did not affect the rate at which the food was eaten. Peptide levels in the rumen were not followed in sheep 2, in view of the large amount present in the casein digest which formed part of the diet. With sheep 2, the less rapid disappearance of free α-amino N and the increased ammonia production when starch and sucrose were omitted was probably due to diminished bacterial growth. With sheep 1, increased levels of α-amino N and ammonia were observed when starch and sucrose were omitted, but the peptide N levels were similar on both diets. These results confirm those of McDonald (1952), who showed that the addition of starch to the rumen lowered the ammonia level there, and are in line with the well-established observation that the utilization of non-protein N by ruminants is improved when fermentable carbohydrate is also fed.

Possible absorption of amino acids from the rumen. Under certain circumstances the level of α-amino N in rumen contents is considerably higher than that of blood. This is almost certainly so when animals are feeding on fresh grass, which contains considerable amounts of free amino acids. The possibility that amino acids might be absorbed from the rumen was investigated by adding comparatively large amounts of casein hydrolysate to the sheep rumen and comparing α-amino N levels in portal and peripheral blood. In a preliminary acute experiment 100 g. of casein hydrolysate in 200 ml. of water (pH 7·0) was added to the rumen of a 50 kg. sheep, and the animal immediately anaesthetized with

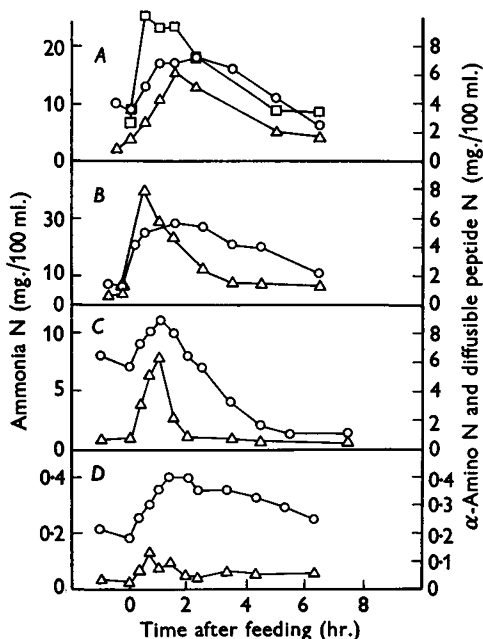

Fig. 1. Rumen concentrations of ammonia (○), α-amino N (△) and diffusible peptide N (□), in sheep 1 (*A*), 2 (*B*), 3 (*C*) and 6 (*D*). Diets are given in the text.

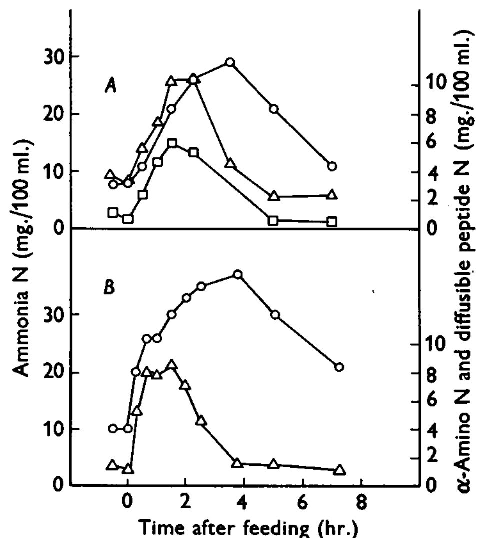

Fig. 2. Concentrations in the rumen of ammonia (○), α-amino N (△) and diffusible peptide N (□), in sheep 1 (*A*) and sheep 2 (*B*) when carbohydrate was omitted from the diets of both sheep on the day of the experiment.

Nembutal (sodium pentobarbitone). The portal vein of the sheep was exposed and 90 min. after placing the casein hydrolysate in the rumen, samples of portal and venous blood were taken simultaneously for α-amino N estimations (plasma and whole blood). No evidence was obtained for any absorption of amino acid into portal blood, since the values for portal blood (whole blood 5·17 and plasma 4·11 mg. of α-amino N/100 ml.) were, in fact, somewhat lower than those for jugular blood (5·65 and 4·27 mg. of α-amino N/100 ml. for whole blood and plasma respectively). Earlier work (unpublished) had indicated that α-amino N levels in simultaneous samples of jugular and carotid blood are similar, both before and after feeding. In a further experiment, portal-blood samples were taken from a conscious sheep by means of a portal-vein catheter (Annison, Lewis & Hill, 1955) before and after the addition of a solution of casein hydrolysate (70 g. in 100 ml. of water) to the rumen. Carotid-blood samples were obtained simultaneously for comparison, the animal possessing a permanently exteriorized carotid artery. The level of α-amino N in the rumen was also followed (Table 1). These results suggest that amino acids are not absorbed from the rumen.

Distribution of α-amino N *in rumen liquor*. The distribution of α-amino N between the micro-organisms and the liquid phase of the rumen contents of sheep 1 before and after feeding was investigated. Samples of strained rumen liquor were centrifuged at 14 000 *g* for 40 min. at 1°, and the original solution and the supernatant were examined for α-amino N after deproteinization, and by the dialysis technique. The results (Table 2) indicate that most of the α-amino N is associated with the rumen particulate matter and that deproteinization results in considerable losses. The 'bound' or internal amino acids of Gram-positive bacteria (Gale, 1953) are liberated from the cells by acid treatment, by boiling or by the action of cetyltrimethylammonium bromide (CTAB), and the action of CTAB on strained rumen liquor was examined. A pre-feed sample of strained rumen liquor (sheep 4) was immediately cooled to 1°, and the following operations were carried out at that temperature. A portion was deproteinized in the usual manner, and a further 50 ml. was treated with CTAB (100 mg.) and allowed to stand for 30 min. before 20 ml. was deproteinized as before. Further portions (25 ml.) of untreated and CTAB-treated strained rumen liquor were centrifuged at 14000 *g* for 40 min., and α-amino N was determined on the supernatant. The residues after centrifuging were washed twice with 40 ml. of O_2-free 0·1 M phosphate buffer containing 0·02 % of Na_2S, then resuspended in water and dialysed as described earlier to allow the diffusible α-amino N to be determined. In addition, the α-amino N contents of CTAB-treated and untreated sheep rumen liquor were determined by the dialysis method. The increased liberation of α-amino N from the micro-organisms after CTAB treatment is apparent from the results shown in Table 3.

To examine the amount of α-amino N associated with a known quantity of microbial N, estimations of α-amino N and Kjeldahl N were made on pre-feed samples of strained rumen liquor from sheep 1 and 4 before and after high-speed centrifuging (14000 *g*, 40 min.). The samples were not treated with CTAB. The difference in Kjeldahl N levels between the original strained rumen liquor and the supernatant is largely a measure of the microbial N, although no doubt some N is contributed by food particles. The results (Table 4) showed that about 2–3 % of the N of the micro-organisms is free α-amino N. This was confirmed by examining the free α-amino N content of well-washed suspensions of rumen bacteria from sheep 1 and 4, immediately before and 3 hr. after feeding. The washed suspensions were prepared as described by Sijpesteijn & Elsden (1952) except that the final suspensions were made in water. All operations were carried out at 1°. The final suspensions were heated at 100° for 15 min. to liberate 'bound' amino N (Gale, 1947) before dialysis in the usual manner. Determination of diffusible α-amino N and peptide N, in relation to the total N of the final suspensions (Table 5), indicated that appreciable amounts of these materials remained associated

Table 1. *Levels of α-amino* N *in portal and carotid-artery blood plasma after the administration of casein hydrolysate to the sheep rumen*

Time after administration (hr.)	α-Amino N levels (mg./100 ml.)		
	Rumen contents	Portal blood	Carotid blood
0	0·86	3·48	3·04
1	83·7	3·18	2·94
1½	—	2·54	2·91
4½	18·8	2·02	1·93

Table 2. *α-Amino* N *levels in rumen liquor of casein-fed sheep* (1) *determined before and after high-speed centrifuging of the liquor*

Sample	Method of treatment	α-Amino N (mg./100 ml.)
Pre-feed	SRL* deproteinized	1·29
	Supernatant deproteinized	0·13
	SRL dialysed	2·19
	Supernatant of SRL dialysed	0·28
Post-feed	SRL deproteinized	2·09
	Supernatant deproteinized	0·67
	SRL dialysed	3·67
	Supernatant of SRL dialysed	0·78

* SRL: strained rumen liquor.

with the cells after thorough washing. These diffusates were examined for amino acids by paper chromatography before and after hydrolysis (see below).

Table 3. *α-Amino* N *levels in rumen liquor of casein-fed sheep* (1) *determined before and after CTAB treatment*

Sample	Method of treatment	α-Amino N (mg./100 ml.)
Untreated SRL*	Deproteinized	1·41
	Centrifuged, supernatant deproteinized	0·72
	SRL dialysed	1·51
	Washed suspension dialysed	0·56
CTAB-treated SRL	Deproteinized	1·53
	Centrifuged, supernatant deproteinized	1·27
	SRL dialysed	2·02
	Washed suspension dialysed	0·73

* SRL: strained rumen liquor.

Table 4. *Total* N *and α-amino* N *levels in rumen liquor of casein-fed sheep* 1 *and* 4, *before and after high-speed centrifuging of liquors*

Rumen liquors were centrifuged at 14000 *g* for 40 min. Samples were taken 24 hr. after feeding.

Sample	Kjeldahl N (mg./100 ml.)	α-Amino N (mg./100 ml.)
Sheep 1, SRL*	76	2·42
Sheep 1, SRL supernatant	21	0·17
Sheep 4, SRL	101	2·61
Sheep 4, SRL supernatant	31	1·21

* SRL: strained rumen liquor.

Table 5. *Total* N, *α-amino* N *and diffusible peptide* N *content of well-washed suspensions of rumen micro-organisms from sheep* 4 *and* 7

Samples taken before and 4 hr. after feeding.

Sample	Total N (mg. of N/ 100 ml.)	α-Amino N (mg. of N/ 100 ml.)	Peptide N (mg. of N/ 100 ml.)
Sheep 4, pre-feed	182	1·65	1·51
Sheep 4, post-feed	198	2·17	0·68
Sheep 7, pre-feed	214	3·70	2·09
Sheep 7, post-feed	205	1·95	0·66

Table 6. *Total* N *and α-amino* N *content of washed suspensions of rumen micro-organisms graded for size by differential centrifuging*

Fraction	α-Amino N (mg./100 ml.)	Total N (mg./100 ml.)	Ratio: Total N / α-Amino N
Whole population	1·1	54·0	49·1
1	0·71	34·0	47·9
2	1·20	58·2	48·5
3	1·77	87·3	49·4
4	0·46	35·9	78·0

The microbial population of a pre-feed sample of rumen contents from casein-fed sheep 4 was fractionated by differential centrifuging into four parts. The fractions were obtained by centrifuging successively 80 ml. of rumen liquor, under the following conditions: 1, 1000 *g* for 3 min.; 2, 2000 *g* for 4 min.; 3, 2500 *g* for 5 min.; 4, 14 000 *g* for 40 min. A sample of the whole population (including Protozoa) was separated by centrifuging the strained rumen liquor (20 ml.) at 14 000 *g* for 40 min. Each fraction was washed twice in 0·1 M phosphate buffer and finally resuspended in water (15 ml.). The total N and α-amino N content of the suspensions (Table 6) suggested that the 'bound' α-amino N was not associated with a fraction of any particular size.

Analysis of the amino acids liberated from washed suspensions of rumen organisms. The amino acids present in the diffusates of the washed suspensions examined above (Table 5) were examined by paper chromatography before and after hydrolysis. Each sample contained a large number of amino acids, but although no single amino acid was present in overwhelming proportions, large well-defined spots identified as valine and leucine/isoleucine were obtained in each case. Aromatic amino acids appeared to be absent, and there were no qualitative differences in the chromatograms obtained from any one suspension before and after hydrolysis. Proline, glutamic acid, aspartic acid, alanine, serine, threonine and arginine were provisionally identified by their R_F values.

In vitro *degradation of proteins by washed suspension of rumen bacteria*

Degradation of casein. The breakdown of casein by washed suspensions of rumen organisms from the sheep fed on hay, groundnut and casein (3, 7 and 4 respectively) was examined. Identical amounts of casein (250 μmoles of N in 2 ml. of solution) were used in each experiment. The time course of liberation of volatile fatty acids, ammonia, free α-amino N and diffusible peptide N at pH 7·0 was followed (Fig. 3) and substantial degradation of the casein occurred in each case. Diffusible peptide N was rapidly liberated in each instance, indicating the marked proteolytic activities of the washed suspensions from each sheep irrespective of diet. The proportion of ammonia in the split products resulting when casein was incubated with the organisms from the sheep fed on hay (Fig. 3, *A*) was much lower than those observed when washed suspensions from the sheep fed on casein and groundnut were examined. In each instance the rate of liberation of free α-amino N was similar to or exceeded that of ammonia production, which is in marked contrast to the situation *in vivo*, where the rumen concentrations of free α-amino N are always considerably less than those of ammonia. This

difference probably results from the partial loss of deaminating power of the organisms which, it has been observed, invariably occurs during the preparation of the suspensions (Lewis, 1955), and it is also likely that *in vivo* the rumen flora rapidly utilize amino acids. As expected, roughly equivalent amounts of volatile fatty acids and ammonia were produced during the incubation of casein. Values obtained for diffusible peptide N levels were somewhat lower than the true figures, since some destruction of amino acids occurs under the conditions employed for acid hydrolysis. Adequate controls are essential in this type of experiment, and in each case analyses were made on suspensions incubated without casein for similar times, and suitable corrections made to the experimental results. Thus when a well-washed suspension of rumen organisms from a sheep fed on casein was incubated alone for 3 hr. under the usual experimental conditions, 7% of the total N of the suspension appeared as ammonia. The α-amino N content of the organisms slowly decreased during this period, but this source of N accounted for only about 10% of the ammonia. The time course of casein degradation by washed suspensions of rumen bacteria from casein-fed sheep 4, in the presence of toluene (0·5 ml. in each Lewis–Elsden flask) which inhibits deamination (Warner, 1955), was also examined (Fig. 3, *D*), and these results can be compared with those obtained in the absence of toluene (Fig. 3, *C*). Rapid liberation of peptides occurred and the suppression of deamination resulted, as expected, in the production of less ammonia and considerably more free α-amino N.

The volatile fatty acids produced by the incubation of casein with a washed suspension from casein-fed sheep 4, the results of which are shown in Table 7, were examined by gas chromatography. The molecular proportions of the individual volatile fatty acids produced by the control suspension and the suspension incubated with casein are shown together with the figures for the volatile fatty acids produced by the breakdown of casein, calculated on the assumption that the breakdown of endogenous material is not influenced by the presence of the casein.

The influence of pH on the relative amounts of products formed by the degradation of casein incubated with a washed suspension of rumen

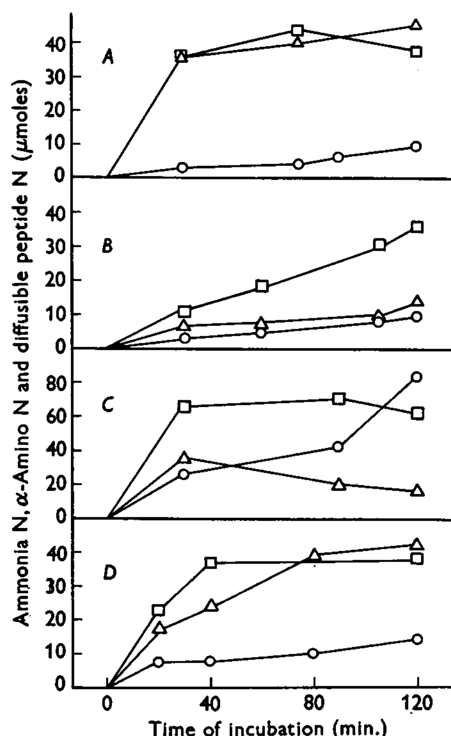

Fig. 3. Time course of liberation of ammonia (○), α-amino N (△) and diffusible peptide N (□) from casein when incubated with washed suspensions of rumen organisms from sheep 3 (*A*), 7 (*B*), 4 (*C*) and from sheep 4, in the presence of toluene (*D*).

Table 7. *Volatile fatty acids produced by the incubation of casein with a washed suspension of rumen organisms from a casein-fed sheep* (4)

Casein (200 μmoles of N) was incubated for 2 hr. with the washed suspension of organisms (0·48 mg. of N/ml.). VFA: volatile fatty acids.

		Molecular percentages of VFA						
Sample	VFA content (μmoles)	Formic	Acetic	Propionic	*iso*Butyric	*n*-Butyric	*iso*Valeric + 2-methyl-butyric	*n*-Valeric
Original suspension	18	0	81	12	1	2	3	1
Suspension incubated alone	72	0	81	10	2	3	3	1
Suspension incubated with casein	120	0	73	13	3	6	4	1
Calculated VFA production from casein	48	0	61	19	4	10	5	1

bacteria from sheep 4 is shown in Fig. 4, *A*. Phosphate buffers (0·1 M) prepared from M-NaH_2PO_4 and K_2HPO_4 were employed, and pH determinations were made on control flasks set up in parallel with the experimental flasks and containing casein, phosphate buffer and washed suspension in similar proportions. The results indicated that the overall degradation of casein was relatively insensitive to pH in the range 6·0–8·0, but peptide breakdown and amino acid deamination occurred most rapidly at pH 6·9. Peptide splitting was probably stimulated at pH 6·9 by the removal of the reaction products by deamination, since, when the experiment was repeated in the presence of toluene (0·5 ml. in each flask), peptide production was maximal at pH 6·9, but peptide degradation as measured by free α-amino N production was similar over the range of pH 6·0–7·0 (Fig. 4, *B*).

Comparison of rates of degradation of different proteins in vitro. The breakdown of casein, bovine albumin, arachin, zein, wheat gluten and soya protein (α-protein) by washed suspensions of rumen bacteria from sheep 3, 4 and 7, fed on hay, casein and groundnut meal respectively, was examined. The six proteins were incubated simultaneously with each suspension. The soluble proteins, casein and bovine albumin, were examined in aqueous solution, but the partially soluble materials were suspended in water. These proteins (250 μmoles of N each) were

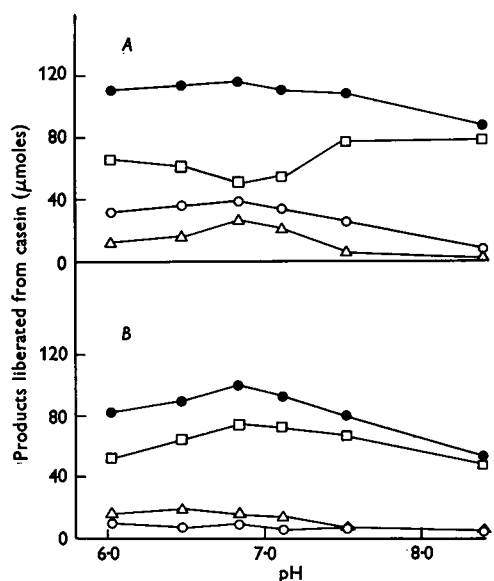

Fig. 4. Influence of pH on the production of ammonia (○), α-amino N (△) and diffusible peptide N (□) when casein was incubated with washed suspensions of rumen organisms alone (*A*), and in the presence of toluene (*B*). The extent of total degradation measured as the sum of the nitrogenous breakdown products is also given (●).

Table 8. *Products obtained by the incubation of different proteins with washed suspensions of rumen organisms from sheep maintained on different diets*

	NH_3 (μmoles)			α-Amino N (μmoles)			Peptide N (μmoles)			Volatile fatty acids (μmoles)			Total degradation (μmoles of N)		
Diet of sheep ...	Hay	Casein	Ground-nut	Hay	Casein	Ground-nut	Hay	Casein	Ground-nut	Hay	Casein	Ground-nut	Hay	Casein	Ground-nut
Substrate															
Casein	27	50	27	34	19	45	35	43	25	33	43	33	96	112	97
Bovine albumin	14	10	0	9	0	0	0	13	1	4	4	0	23	23	1
Arachin	25	22	19	16	10	11	14	22	11	16	19	20	55	54	42
Zein	2	0	5	10	0	0	0	13	3	1	0	10	12	13	8
α-Protein	19	18	19	23	8	11	22	26	25	21	20	22	64	52	56
Wheat gluten	5	0	8	12	0	0	—	16	6	8	0	16	—	16	14

incubated for 2 hr. under the experimental conditions described earlier, with washed suspensions containing about 1 mg. of bacterial N/ml. The products, volatile fatty acids, ammonia, α-amino N and diffusible peptide N, were measured and the results are shown in Table 8. The total degradation of each protein examined, i.e. the sum of the N-containing split products (Table 8) were strikingly similar in most cases for each protein when incubated with the different washed suspensions. An exception was purified bovine albumin, which, although slightly degraded on incubation with washed suspension of rumen organisms from sheep fed on hay and casein, was not attacked by a washed suspension from the sheep receiving groundnut cake. It seemed possible that the albumin contained an impurity which inhibited proteolytic activity, but when mixtures of casein and albumin were incubated with washed suspensions of rumen organisms from the casein-fed sheep the protein degradation was 10–20% greater than that which occurred when similar quantities of the proteins were examined separately under identical conditions.

DISCUSSION

The demonstration of measurable quantities of amino acids and peptides in rumen contents is further evidence that proteolysis is the first step in the digestion of protein in the rumen. This is well illustrated by the results of analyses of rumen contents after the ingestion of rations containing protein but not free amino acid or peptide (Fig. 1, *A*). The failure of the α-amino nitrogen level to rise above about 10 mg./100 ml., and its rapid fall 2–3 hr. after the cessation of feeding are presumably due to the rapid uptake or deamination of the amino acids by the rumen micro-organisms, since no evidence was obtained for their absorption from the rumen into the portal system.

Additional evidence of the proteolytic activity of rumen contents was obtained by incubating a number of proteins with washed suspensions of rumen bacteria, when casein, arachin and soyabean protein were found to be extensively degraded, but bovine albumin, wheat gluten and zein were only slightly attacked (Table 8). Casein and arachin were shown by Annison *et al.* (1954) to suffer extensive degradation to ammonia in the sheep rumen, and the slow digestion of zein was demonstrated by McDonald (1954). Although no results have been reported on the digestion of albumin in the rumen, blood meal was found by Pearson & Smith (1943) to be only slowly attacked. When the total degradation of casein by the washed suspensions from the sheep maintained on three different diets is considered, the quantities of casein degraded are more similar than the ammonia production would suggest. El-Shazly (1952*b*) found that sheep fed on casein develop rumen flora with vigorous deaminating properties. In general the results indicate that washed suspensions from sheep fed on different diets are of similar proteolytic activity, but that casein feeding results in the development of rumen flora with vigorous deaminating power towards casein. Similar conclusions were reached by Warner (1955).

Convincing evidence has been obtained that the value of casein to the ruminant animal varies inversely with the extent to which the protein is degraded by microbial action in the rumen (Chalmers *et al.* 1954; Chalmers & Synge, 1954*b*). If the ingested protein is of low biological value, however (unlike casein), its degradation and resynthesis to microbial protein of high biological value might be advantageous. Thus although in general proteins which are readily degraded to ammonia in the rumen are unlikely to prove satisfactory as ruminant foodstuffs, since much of the ammonia is absorbed, converted into urea and excreted, each protein must be considered separately. The major factors in rumen metabolism which influence protein value are: (1) ammonia absorption; (2) the extent of synthesis of nitrogenous compounds of low or negligible value to the host, e.g. nucleic acids; (3) the relative biological values of microbial proteins and feed proteins, and the proportions of these two which finally become available for digestion (McDonald, 1952).

The association of a substantial part of the free α-amino nitrogen of rumen contents with the microbial population implies that, except after feeding, when a substantial rise occurs, the normal level of α-amino nitrogen in the liquid phase of rumen contents does not reflect the amounts of amino acids available for reactions in the rumen. Similarly, detailed analyses for amino acids of supernatants obtained by the centrifuging of rumen contents are of limited interest, since it is probable that the amino acids associated with the microbial cells and rumen liquid are in dynamic equilibrium. When the reactions of amino acids incubated with suspensions of rumen organisms are examined it would seem desirable to follow the fate of added substrate in addition to the formation of end products. The amino acids associated with rumen organisms are not 'bound' in a manner strictly similar to that which obtains with certain Gram-positive bacteria such as *Staphylococcus aureus* (Gale, 1953), since although a substantial amount is retained after several washings with buffer, most of the α-amino nitrogen is released by dialysis for 24 hr. at 1°.

The behaviour of substrates incubated with washed suspensions of rumen bacteria provides useful information on the degradative reactions which probably occur *in vivo*. When protein

degradation is examined by this procedure, the system is complex since (*a*) the bacterial population is mixed, and the individual organisms are in different stages of growth, and (*b*) many enzymes are successively involved in the breakdown of protein to volatile fatty acids and ammonia. The isolation of individual organisms involved in nitrogen metabolism in the rumen is in progress, and the proteolytic enzyme systems will be examined in some detail. Studies on the various aspects of nitrogen metabolism in the rumen may ultimately provide information which will allow the value of a particular ration to be predicted without recourse to large-scale feeding trials.

SUMMARY

1. Measurable amounts of α-amino nitrogen (0·3–1·5 mg./100 ml.), and of α-amino nitrogen which is liberated on hydrolysis of the diffusible peptides (0·2–1·0 mg./100 ml.), are present in rumen contents under resting conditions. The concentrations of these constituents may show a five- to ten-fold increase during and immediately after feeding.

2. The analysis for α-amino nitrogen of portal and arterial blood taken from a sheep with a casein hydrolysate placed in its rumen suggested that amino acids are not absorbed from the organ.

3. The rates of disappearance of ammonia and amino acids from the sheep rumen after the feeding of casein or casein hydrolysate were increased in the presence of carbohydrate.

4. Much of the free α-amino nitrogen of whole rumen contents is associated with the micro-organisms present.

5. The time course of liberation of diffusible peptides and amino acids from casein, and the production of ammonia and volatile fatty acids was followed when the protein was incubated with washed suspensions of rumen organisms.

6. The *in vitro* degradation of casein, bovine albumin, arachin, zein, wheat gluten and soya protein, by washed suspensions of rumen organisms from sheep fed on diets based on hay, casein and groundnut, was examined. Casein, arachin and soya protein were readily degraded, but bovine albumin, zein and wheat gluten were less extensively attacked.

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