The Role of Ammonia in Ruminal Digestion of Protein

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(Received 3 August 1951)

In a previous communication attention was drawn to the occurrence in the rumen of the sheep of significant concentrations of ammonia (McDonald, 1948*a*). It was shown that urea was present in the saliva of sheep, and that this was one source of ammonia, since Lenkeit & Becker (1938) had demonstrated a high urease activity of the micro-organisms of the rumen. Evidence was also presented to prove that ammonia was absorbed from the rumen into the portal blood stream, and, since only mere traces of ammonia could be detected in the blood of the general circulation, it was concluded that the absorbed ammonia was converted by the liver into urea; thus a circulation of nitrogen via the saliva, ruminal ingesta and liver could be envisaged. Further observations which indicate the importance of ammonia in the turnover of nitrogen in the rumen are reported in this paper.

EXPERIMENTAL

In all experiments the sheep were fed once daily, but were allowed continuous access to water. The simple diets used were always adequate to maintain the animals in robust health.

The sheep used in these experiments were provided with rumen fistulae, to enable sampling of the rumen contents. No means are available for taking a representative sample of the rumen contents; this difficulty, which has been discussed by Pearson & Smith (1943), is due to the fact that the rumen contents are heterogeneous and contain large fragments of plant material which are not uniformly distributed throughout the entire mass of ingesta. In order to avoid this difficulty, the analyses recorded here were made on the fluid obtained from the rumen contents after straining through muslin. The object was to obtain a sample of the rumen liquor which would contain the soluble nitrogenous constituents and the micro-organisms, but not the dietary plant fragments. This expedient was not entirely satisfactory, since some of the smallest plant fragments passed through the muslin, while large numbers of micro-organisms were retained with the plant residues. It was, however, observed that samples of rumen liquor obtained from widely separated points in the rumen showed the same composition. It is also probable that the smallest plant fragments were those which had been most thoroughly attacked by the ruminal bacteria and hence contributed but little protein to the mixture. The most unsatisfactory feature was the loss of micro-organisms on the plant residues and thus the reduction of the concentration of protein in the rumen liquor.

The rumen liquor will also contain some protein from the saliva, but the concentration of protein in saliva is small in

comparison with that of the rumen liquor, and since the salivary protein would also be susceptible to digestion in the rumen, any error from this source was presumed to be negligible.

With these reservations, it may be taken that the protein of rumen liquor consists essentially of the protein of the contained micro-organisms. Indirect evidence in favour of this conclusion was found in the very high concentration of protein in the dry matter of the rumen liquor; an average of nine analyses gave the value of about 50% protein (protein N $\times 6.25$) in the dry matter after allowance was made for the content of ash and volatile fatty acids. These reservations do not, however, preclude the use of analyses of rumen liquor to observe the trend of changes in the distribution of nitrogen in the rumen in relation to time after feeding, and the procedure is certainly valid for the soluble non-protein nitrogen (N.P.N.) constituents; only limited conclusions can be drawn from the values obtained for protein N.

Methods

Total nitrogen was estimated by the Kjeldahl method, using the semi-micro technique of Chibnall, Rees & Williams (1943).

Ammonia was estimated by the method of Conway (1947) or by distillation of protein-free filtrates with NaOH-borate buffer at pH 8.5. The two methods gave excellent agreement and were in accord with values obtained by the method of Parnas & Klisieski (1926).

Non-protein nitrogen (N.P.N.) was estimated as the total N in protein-free filtrates obtained either by precipitation with ethanol or with dilute acid. It was found that the protein of rumen liquor, obtained by filtering rumen contents through muslin, could be quantitatively precipitated by dilution (1 to 10) and acidification to approximately pH 2.5.

Protein N was calculated as total N minus N.P.N.; for convenience of description, the N.P.N. was divided into two categories, ammonia N and residual N. The components of the residual N have not been studied.

RESULTS

Meadow-hay diet

Preliminary observations were made with sheep fed an exclusive diet of meadow hay; a graph showing the changes in the distribution of nitrogen in the rumen liquor is given in Fig. 1. The values shown are the averages of three observations on each of two sheep. The main features shown by these curves are as follows. The N.P.N. consists chiefly of ammonia N, while the residual N is always of low concentration. Qualitatively, amino-acids could not be detected by direct ninhydrin tests or by paper chromatography of protein-free filtrates. The rapidity with which the

ammonia accumulates in the rumen after feeding is a reflexion of the high activity of the micro-organisms. Since amino-acids do not accumulate in the ingesta, it is apparent that the rate of uptake of amino-acids by the microbes from the medium exceeds the rate of proteolysis by the proteases formed by them, or that they may in addition actively deaminate free amino-acids.

Fig. 1. Changes in the distribution of nitrogen in the rumen liquor of sheep on a diet of meadow hay. Each point represents the average of three observations on each of two sheep.

The peak in the curve for ammonia concentration does not give an indication of the magnitude of total formation of ammonia since this is removed from the rumen in several ways: by its utilization by bacteria for their growth, by passage in the ingesta from the rumen to the omasum and abomasum, and by direct absorption from the rumen (McDonald, 1948*a*). The relative magnitude of these effects has not yet been estimated with accuracy.

Casein and zein diets

The close association of the changes of concentration of ammonia in the rumen with feeding suggested that part of the ammonia was derived from the proteins of the feed. In order to test this hypothesis, proteins in suspension were added directly to the rumen through a fistula. It was first established that a satisfactory base-line could be obtained by sampling the rumen contents during the period of 16–24 hr. after feeding; the control curve in Fig. 2 shows that during this time the concentration of ammonia in the rumen remains virtually constant; the factors responsible for the accumulation and removal of ammonia have thus come into equilibrium. Casein, gelatin and zein were chosen as representative proteins on the ground that the two former are soluble and readily attacked by proteinases, while the latter is highly insoluble in aqueous media and relatively resistant to proteo-

lysis. The results given in Fig. 2 show clearly that the addition of 25 g. casein to the rumen was followed by a pronounced rise in the ammonia concentration. An approximate estimate indicates that the ammonia nitrogen formed from the casein represented about 20% of the total N added. Since amide N comprises only 9.3% of the total N of casein, the observed rise in ammonia cannot be due solely to the

Fig. 2. Comparisons of the effects on ammonia formation of addition of protein suspensions to the rumen of a sheep; 25 g. of protein were added at the time indicated by the arrow. Control curve, no protein added.

removal of amide groups; it was concluded therefore that deamination as well as deamidation reactions were responsible for the formation of ammonia. This conclusion was supported by the fact that similar results were obtained when a solution of gelatin, which contains only a trace of amide N (Chibnall, 1942), was added to the rumen.

The direct addition of 25 g. zein to the rumen produced no change in the concentration of ammonia N in the rumen. This is clearly a reflexion of the physical properties of the protein which render it so resistant to proteolysis (Laine, 1944). Since zein is in fact digested to a considerable extent in the rumen (McDonald, 1948*b*) it is evident that its rate of digestion is too slow to permit an accumulation of ammonia.

These experiments showed that ammonia could be derived from protein in the feed. Observations were then made on the distribution of nitrogen in the rumen fluid when sheep were fed on partially purified diets in which casein or zein comprised the chief source of nitrogen. The daily ration was composed of protein (casein or zein), 110 g.; starch, 280 g.; cellulose, 250 g.; glucose, 80 g.; molasses, 40 g.; chaffed straw, 150 g. and adjuvants of mineral salts and vitamins A and D; the prepared diets were found to be palatable and the day's ration was consumed within a few hours.

The distribution of nitrogen in the rumen liquor during feeding regimes with these two diets is

shown in Figs. 3 and 4. Again it is clear that the soluble casein is readily attacked with the liberation of ammonia and of other N.P.N. substances (expressed as residual N). The rise in residual N is of comparatively brief duration, and after 2 hr. the

Fig. 3. Changes in the distribution of nitrogen in the rumen liquor of a sheep on a diet in which casein comprised the chief source of nitrogen. Each point represents the average of three observations.

Fig. 4. Changes in the distribution of nitrogen in the rumen liquor of a sheep on a diet in which zein comprised the chief source of nitrogen. Each point represents the average of four observations.

value begins to decline while the level of ammonia N continues to rise for 4 hr. after feeding. In sharp contrast is the effect of feeding zein in the diet; the ammonia falls to extremely low levels after feeding, while the residual N shows no change. This finding can best be interpreted as indicating that the rate of proteolysis of the zein is slower than the capacity of the bacteria to take up the products of proteolysis and that in the presence of readily available sources of energy (in this case, glucose and starch) the organisms use the ammonia as a source of nitrogen for growth; later when the growth rate of the bacteria declines, the rate of formation of ammonia

may exceed the rate of uptake and the concentration of ammonia rises slowly to the pre-feeding level. Further evidence in support of this view is the observation that when starch alone is added to the rumen during the late post-feeding stage there is a steady decline in the concentration of ammonia; the results of an experiment with two sheep are shown in Fig. 5.

Fig. 5. The influence on ammonia concentration in the rumen liquor of addition of starch suspension to the rumen of sheep during the late post-feeding phase.

The relative availability of the two proteins for bacterial growth is illustrated in the marked difference in the levels of protein N in the rumen liquor in spite of the fact that both diets contained the same amount of nitrogen. On the zein diet, the protein N level in the rumen liquor was approximately 55 mg./100 ml. compared with 110 mg./100 ml. on the casein diet.

DISCUSSION

The general purpose of the work reported here was to obtain information, both qualitative and quantitative, on the role of micro-organisms in the digestion of protein in the rumen. It has already been established by Wegner, Booth, Bohstedt & Hart (1940) that the saliva of ruminants contains no proteolytic enzymes and it is well known that the stratified squamous epithelium of the mucosa of the rumen and reticulum possesses no secretory glands. It is therefore evident that digestion of protein in the rumen can be due only to proteolytic enzymes contained in the food or produced by the microbes (protozoa and bacteria) which inhabit this viscus. In order to eliminate the former as a significant factor, preliminary experiments were conducted with diets consisting exclusively of hay, in which the leaf proteins are denatured by the drying of the leaves (Lugg, 1946); under these conditions it was considered that little, if any, proteolysis could be ascribed to surviving plant enzymes. By contrast, the rumen supports an extremely large population of microbes; Van der Wath & Myburgh (1941) recorded protozoal counts exceeding 2 millions/ml. of rumen contents, and

Gall, Burroughs, Gerlaugh & Edgington (1949) found bacterial counts exceeding 50×10^9 /g. of rumen contents. Since this dense population of microbes is maintained in spite of continuous passage of ingesta from the rumen, it is evident that a significant fraction of the nitrogen of the host's diet must be digested and utilized by the microbes for their own growth. The presence of highly active proteinase, considered to be of microbial origin, in the ruminal contents was demonstrated by Sym (1938), who used casein as substrate for *in vitro* tests. There is little reason to doubt that, for all practical purposes, the whole of the digestion of protein in the rumen is effected by micro-organisms.

In the general physiological economy of the ruminant, the essential function of the rumen may be envisaged as the digestion of cellulose and other carbohydrates for which the animal does not produce digestive enzymes; other changes in the rumen can be considered as coincidental to this function. In this respect, the anatomical arrangement of the digestive organs, which provides for the microbial digestion of cellulose prior to the operation of the animal's own enzymic secretions, confers on the ruminant a more efficient mechanism than is found in other herbivores, in which the bacterial degradation of cellulose follows the activity of the gastric and intestinal secretions. The digestion of cellulose requires the provision by the host animal of an environment in which the cellulose-splitting bacteria may thrive; this environment is provided in the rumen with a high degree of regulation (Phillipson, 1946), with the result that a population of bacteria and protozoa in very large numbers is maintained. This population requires a suitable and adequate supply of nitrogen; under normal feeding conditions, most of the nitrogen in the animal's diet will be comprised of protein, but other nitrogenous substances will always be present. For none of the natural feeds are complete analyses available for the distribution of nitrogenous substances; in some cases it is known that amides or free amino-acids contribute to the N.P.N. fraction, but most natural feeds will contain a variety of N.P.N. substances in other forms.

Since so many organisms are capable of using ammonia as the sole or part source of nitrogen for growth, and since ammonia is also the chief nitrogenous end product in the breakdown of proteins by bacteria (Stephenson, 1949), it is scarcely surprising that ammonia should figure so prominently in the nitrogen metabolism of the rumen. Shazly & Synge (1950) have demonstrated the marked capacity of ruminal bacteria to deaminate amino-acids; when suspensions of the washed bacteria were incubated with acid-hydrolysed casein, up to 35% of amino-acid N appeared as ammonia. In addition, the rumen bacteria possess desamidase which

actively produces ammonia from asparagine and glutamine (author's unpublished experiments). It is quite probable that some others of the N.P.N. substances occurring naturally in fodders may be degraded with the formation of ammonia, and to the degree that this occurs, these substances would contribute to the turnover of nitrogen in the rumen in exactly the same way as at least part of the amino-acids and amides. The available data are too inadequate to evaluate these processes.

It has now been established that the ruminant can survive on a diet in which virtually all the nitrogen is supplied in the form of urea (Loosli, Williams, Thomas, Ferris & Maynard, 1949), which is converted in the rumen into ammonia, but it is evident that a normal population of micro-organisms is not maintained in the rumen on such a diet (Gall, Thomas, Loosli & Huhtanen, 1951). It seems likely that many of the ruminal species are exacting in their requirements for amino-acids and that a diet which supplies little or no nitrogen in the form of protein or amino-acids, would lead to the disappearance of the exacting species and the survival or establishment of species which can use ammonia as the sole source of nitrogen.

The formation of ammonia in the rumen leads to two opposing nutritional tendencies. First, since substances such as urea, which are nutritionally valueless to the host, can be converted to ammonia and utilized for growth of bacteria, that is for synthesis of protein, which can be subsequently digested and used by the host, a gain of nitrogen accrues to the host animal. By contrast, the degradation of protein to ammonia, which can be directly absorbed from the rumen, implies a source of loss of nitrogen to the host animal. The interaction of these opposing tendencies is probably a major factor leading to the relative constancy of the biological value of food nitrogen (crude protein) for ruminants (Johnson, Hamilton, Mitchell & Robinson, 1942).

A provisional outline of the main events concerned in the digestion of protein in the rumen may be given as follows. Under ordinary conditions of feeding, the nitrogen entering the rumen will comprise chiefly protein together with varying amounts of non-protein nitrogenous substances as peptides, amino acids, amides, purines, pyrolles, simple bases such as choline and the betaines, inorganic N as ammonia, nitrates and nitrites, and traces of other substances. The nitrogenous bases and amino compounds may be deaminated while nitrates and nitrites are reduced to ammonia (Lewis, 1951). Ammonia is also produced by the degradation of proteins. In addition, small but significant amounts of nitrogen are added to the rumen contents by the saliva, in which the most important component is urea, which is readily converted into ammonia. Ammonia is utilized by the

micro-organisms for growth, together with amino-acids produced by the activity of the bacterial proteases. Protein leaving the rumen by passage in the ingesta to the more distal parts of the gastrointestinal tract consists of a mixture of undigested food protein and the protein of the micro-organisms. The ratio of these two forms of protein in the ingesta leaving the rumen has not yet been determined under any natural feeding conditions, but McDonald (1948*b*) has reported the extent of conversion of zein to microbial protein in sheep fed a partially purified diet. Ammonia is absorbed from the rumen and in part may return to the rumen, after passage through the liver, by secretion as urea in the saliva, while in part it would be excreted in the urine as urea. Some of the nitrogen utilized by the ruminal micro-organisms for growth would appear as nucleic acids (and other non-protein substances) which are probably of very limited, if any, value to the host animal. The biological value of protein or of any

other nitrogenous substance in the diet of a ruminant will be determined, in part, by the degree to which it is attacked and utilized by the ruminal micro-organisms.

SUMMARY

1. In the rumen fluid, ammonia constitutes the main component of the non-protein nitrogen, when the animal is fed natural diets or a diet in which casein is the main source of nitrogen. The insoluble protein, zein, is only slowly digested in the rumen.

2. Indirect evidence suggests that ammonia represents an important intermediate in the digestion of dietary protein and its utilization by the symbiotic micro-organisms of the rumen for their growth.

3. The implications of these observations on the role of micro-organisms in the digestion of protein in the rumen are discussed.

REFERENCES

- Chibnall, A. C. (1942). *Proc. roy. Soc. B*, **131**, 136.
 Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
 Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby, Lockwood and Son Ltd.
 Gall, L. S., Burroughs, W., Gerlaugh, P. & Edgington, B. H. (1949). *J. Animal Sci.* **8**, 441.
 Gall, L. S., Thomas, W. E., Loosli, J. K. & Huhtanen, C. N. (1951). *J. Nutrit.* **44**, 113.
 Johnson, B. C., Hamilton, T. S., Mitchell, H. H. & Robinson, W. B. (1942). *J. Animal Sci.* **1**, 236.
 Laine, T. A. (1944). *Ann. Acad. Sci. fenn. A ii*, no. 11.
 Lenkeit, W. & Becker, M. (1938). *Z. Tierernähr.* **1**, 97.
 Lewis, D. (1951). *Biochem. J.* **48**, 175.
 Loosli, J. K., Williams, H. H., Thomas, W. E., Ferris, F. H. & Maynard, L. A. (1949). *Science*, **110**, 144.
 Lugg, J. W. H. (1946). *Aust. Chem. Inst. J. Proc.* **13**, 88.
 McDonald, I. W. (1948*a*). *Biochem. J.* **42**, 584.
 McDonald, I. W. (1948*b*). *J. Physiol.* **107**, 21 P.
 Parnas, J. K. & Klisiecki, A. (1926). *Biochem. Z.* **173**, 224.
 Pearson, R. M. & Smith, J. A. B. (1943). *Biochem. J.* **37**, 142.
 Phillipson, A. T. (1946). *Vet. Rec.* **58**, 81.
 Shazly, K. & Synge, R. L. M. (1950). *Int. Physiol. Congr. Copenhagen*. Abstr. p. 445.
 Stephenson, M. (1949). *Bacterial Metabolism*, 3rd ed. London: Longmans, Green and Co.
 Sym, E. A. (1938). *Acta Biol. exp., Varsovie*, **12**, 192.
 Van der Wath, J. G. & Myburgh, S. J. (1941). *Onderstepoort J. vet. Sci.* **17**, 61.
 Wegner, M. I., Booth, A. N., Bohstedt, G. & Hart, E. B. (1940). *J. Dairy Sci.* **23**, 1123.

Effect of Haemoglobin and other Nitrogenous Compounds on the Respiration of the Rhizobia

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(Received 7 August 1951)

Kubo (1939) established that the red pigment in leguminous root nodules is a haemoprotein with absorption characteristics almost identical with animal haemoglobin, and that the addition of the pigment to a suspension of soybean organisms enhanced their oxygen uptake on succinate. In a paper published in 1943 by Kasugai, Kubo & Tsujimura, but only recently available, these initial obser-

vations were extended to include pure cultures of the root nodule and other bacteria. The stimulation by haemoglobin was blocked by hydroxylamine, which combines only with ferric iron; this inhibition is surprising if the haemoprotein were merely oxygenated and deoxygenated, for the iron would then remain in the ferrous state. Despite this observed effect of hydroxylamine, Kasugai *et al.* concluded