RUMINAL FLORA STUDIES IN THE SHEEP

V. THE AMINO ACID COMPOSITION OF RUMEN BACTERIAL PROTEIN

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Summary

Fifteen amino acids were determined, by paper chromatography, on the protein preparations of each of two bulk samples of rumen bacteria from sheep fed under "dry" and "green" feed conditions.

Very similar patterns of amino acid distribution were found for the two samples and no outstanding differences were apparent between them and the values recorded in the literature for a range of microorganisms. The arginine, histidine, tryptophane, and glutamic acid contents were, however, higher in the rumen bacterial proteins and the isoleucine content somewhat lower than those recorded for other bacteria.

A comparison of rumen bacterial protein with whole egg protein is made which indicates that it is inferior as a source of leucine, threonine, and phenylalanine and markedly inferior as a source of methionine and isoleucine. Methionine and isoleucine are considered to be the "limiting" amino acids in the nutritional value of rumen bacterial protein.

These findings are discussed in relation to the utilization of nitrogen and the biological value of proteins to ruminants.

I. INTRODUCTION

Indisputable evidence that the bacteria of the rumen achieve considerable protein synthesis from non-protein nitrogen materials in the diet is now available from many sources. Even where the dietary nitrogen is entirely protein, appreciable conversion of this protein to bacterial protein is apparent from the work of Johnson *et al.* (1942), McDonald (1948*a*), and Moir and Williams (1950). A variable proportion of the nitrogen ultimately utilized by the ruminant is bacterial protein. For this reason several studies of the nutritive value of rumen bacterial protein have been made (Johnson *et al.* 1944; McNaught *et al.* 1950; Reed, Moir, and Underwood 1949). The last-named workers found the protein of their bacterial preparations, as judged by nitrogen-balance studies with growing rats, to be low in digestibility but relatively high in biological value. The biological value was, in fact, not significantly different from that found for casein fed under the same conditions, namely 78-80.

The biological value of a protein, determined under appropriately standardized conditions, provides a very useful indication of the overall nutritive value of the digestible portion of that protein to the young, growing, non-

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ruminant mammal. It does not, however, tell very much of the assemblage of amino acids present or of the specific amino acid deficiencies which may be limiting its usefulness to the animal. Actual amino acid analyses are valuable for this purpose. A beginning in this connection was made by Johanson, Moir, and Underwood (1949) and Reed, Moir, and Underwood (1949). These workers estimated, by the differential oxidation method of Lugg (1938), the cyst(e)ine and methionine contents of the protein of the mixed rumen bacteria samples used for the biological value determinations mentioned in the previous paragraph. They found the rumen bacterial protein to be surprisingly rich in cyst(e)ine, compared with almost all other food or microorganism protein studied, and moderately rich in methionine compared with many of them. The methionine content was, however, much lower than that of whole egg protein and slightly lower than that of muscle protein or casein (Block and Mitchell 1946). Since casein has been shown to be mildly deficient in methionine (Beadles *et al.* 1933; Kik 1938) and rumen bacterial protein has a biological value similar to casein it was tentatively concluded that methionine was the limiting amino acid. The possibility remained that other essential amino acids were also involved. These could only be revealed by much more complete studies in which all the essential amino acids were determined.

Several groups of workers have examined the protein of a range of micro-organisms, including yeasts, actinomycetes, bacteria, and moulds, grown under a variety of cultural conditions. The results of these investigations have been critically assessed by Lugg (1949) and by Block and Bolling (1951) and will not be discussed at this point. Apart from the amino acid values of rumen material obtained from sheep fed urea as the entire N source (Loosli *et al.* 1949), and the cyst(e)ine and methionine values mentioned earlier, no data on the general amino acid make-up of the mixed bacterial protein from the rumen have, as far as is known, been published.

The methods and results of such studies are presented in this paper. They involve the quantitative determination, by means of paper chromatography, of 15 amino acids in the protein of bacteria obtained from the rumen of sheep selected to represent two types of feed conditions.

II. Materials and Methods

(*a*) *Collection and Preparation of Rumen Bacteria*

Details of the collection and preparation of the samples of rumen bacteria are given by Reed, Moir, and Underwood (1949). Two bulk samples were obtained from slaughter-house sheep; one from sheep which had come from dry feed conditions and the other from sheep brought from green grazing. These are referred to throughout as the "dry-fed" and the "green-fed" samples. Each sample was free from protozoa and feed particles and the dry material contained 47.6 per cent. crude protein ($N \times 6.25$) in the "green-fed" and 50.9 per cent. crude protein on the dry basis in the "dry-fed."

(*b*) *Determination of the Amino Acids*

(i) *Protein Preparations.*—Representative 10-g samples were taken from each of the dried bacterial preparations and ground to pass a 0.5-mm sieve. "Whole" protein preparations were made from the ground samples by the method of Lugg (1939) as modified by Lugg and Weller (1944). Nitrogen determinations were made on the ground samples and on the "whole" protein preparations by the micro-Kjeldahl method.

(ii) *Protein Hydrolysis.*—For the estimation of tryptophane, alkaline hydrolysis of the protein preparations was carried out with 5N NaOH in sealed test tubes at 100°C for 24 hr. Following filtration the solution was neutralized with H_2SO_4 and made up to a volume of 20 ml. This neutralized hydrolysate was desalted prior to the chromatographic separation of the amino acids by the use of the electrolytic desalting apparatus designed by Consden, Gordon, and Martin (1947), except that the carbon anode was replaced with platinum (de Verdier and Agren 1948). Tests with standard tryptophane solutions gave a 91 per cent. recovery of tryptophane by this method. The final desalted solutions were then concentrated in a vacuum desiccator.

Acid hydrolysis was used for the rest of the amino acids determined. The protein preparations were hydrolysed with freshly distilled constant boiling HCl (5.7N) in sealed test tubes at 105°C for 24 hr. The quantity of protein preparation taken (200-500 mg) was such as to give a final concentration of approximately 5 mg N/ml with an acid : protein ratio of 10 ml/g. After filtering off the humin formed, the excess acid was removed by evaporation under reduced pressure. The resulting amino acid deposit was taken up in 5 ml of distilled water, slightly acidified to retain the less soluble amino acids in solution.

(iii) *Chromatographic Technique.*—The general methods adopted for the paper chromatographic separation of the amino acid constituents were based on the report of Consden, Gordon, and Martin (1944). For descending solvent movement air-tight galvanized iron tanks were used, the filter paper being suspended from stainless steel troughs. For ascending solvent migration, cylindrical glass museum jars were used in the manner of Williams and Kirby (1948). Whatman No. 4 filter paper was used throughout. Phenol was purified by the method of Williams and Kirby (1948), collidine by the method of Partridge (1948), and alcohols by fractional distillation. Water-saturated solvents were allowed to come to equilibrium until clear solutions were obtained at the chromatographic temperature. To avoid losses of amino acids associated with excessive heating in the presence of solvents (Fowden and Penny 1950) phenol was removed from the chromatogram by ether washing, collidine by drying in a current of air at 50°C, and alcohols by air-drying at room temperature. The dried papers were heated for a further 15 min at 100°C and the positions of the amino acids marked out under ultraviolet light (Phillips 1948).

Methionine, leucine, isoleucine, and phenylalanine were satisfactorily separated by the method of Work (1949) by uni-dimensional runs in the descending manner with water-saturated amyl alcohol as the solvent. The remaining amino acids were separated by two-dimensional runs using two solvent systems, namely

the descending method of Consden, Gordon, and Martin (1944) with water-saturated phenol (1 per cent. NH_3) and collidine, and the ascending method of Boissonnas (1950) with phenol-water (7 : 3) and propanol-water (7 : 3).

(iv) *Amino Acid Estimation.*—Quantitative estimations of the amino acids were based on the reports by Fowden (1951) and Boissonnas (1950), who successfully adapted the ninhydrin reagent of Moore and Stein (1948) to a spectrophotometric estimation from chromatograms. Standard reference graphs, relating amino acid nitrogen to optical density, were drawn up for each amino acid by the use of standard amino acid solutions. The linear plot obtained was shown to be reproducible with an accuracy of ± 2 per cent. By reference to this standard curve quantitative estimations were obtained from the chromatograms with an accuracy assessed at ± 5 per cent.

III. Results

(*a*) *Whole Protein Preparations*

The moisture, total nitrogen, coagulable nitrogen, and protein nitrogen contents of the rumen bacteria samples, together with the nitrogen data for their "whole" protein preparations, are given in the paper by Reed, Moir, and Underwood (1949). The total nitrogen content of the bacteria from the sheep on dry feed is slightly higher (8.13 per cent.) than that of the "green-fed" sample (7.60 per cent.) but the reverse is true of their protein preparations. These are 8.78 and 9.62 per cent. for the "dry-fed" and "green-fed" samples respectively. Both bacterial samples were found to contain considerable amounts of non-protein nitrogen, and in the green-fed samples, of extractable carbohydrates which were removed during the separation of the protein preparations.

(*b*) *Loss of Amino Acids during Hydrolysis*

A variable destruction of amino acids during acid hydrolysis of protein materials, particularly where they contain carbohydrate, has been reported by many workers. In spite of this, the results of amino acid determinations on such materials are frequently presented as if no losses occurred.

The results obtained in the present study are similarly presented, not only to facilitate comparison with those of other workers but also because of the difficulties of assessing the magnitude of these losses. It is probable that no appreciable error is involved, by so doing, with threonine, alanine, phenylalanine, leucine, isoleucine, tryptophane, glycine, glutamic acid, and aspartic acid, as these are little affected by hydrolysis in the presence of carbohydrate (Block and Bolling 1951). With arginine, histidine, lysine, methionine, tyrosine, and valine, on the other hand, there is little doubt that results so presented are too low. Substantial hydrolytic losses have been obtained with these amino acids in an experiment conducted in this laboratory. The actual losses and the conditions of the experiment are being published elsewhere (Holmes 1953) since they do not relate directly to the rumen bacterial protein preparations under investigation. It is considered essential, however, that this point should be borne in mind for a proper appreciation of the results.

(c) *Amino Acid Composition of Rumen Bacterial Protein*

The results of the amino acid determinations on the protein preparations of the two samples of rumen bacteria, expressed as percentage amino acid N of protein N, are presented in Table 1, together with some representative figures on other bacteria obtained from the literature. The composition of a rumen material (Loosli *et al.* 1949) is also included. As urea is the sole source of dietary N, the protein present must be largely microbial, although salivary proteins are probably included. Many other individual amino acid figures could be quoted but those given are sufficiently complete to permit satisfactory

TABLE 1

AMINO ACID COMPOSITION (PERCENTAGE AMINO ACID N OF PROTEIN N) OF THE PROTEINS OF RUMINAL AND OTHER BACTERIA COMPARED WITH WHOLE EGG PROTEIN

Amino Acid	Rumen Bacteria, "Dry-Fed"	Rumen Bacteria, "Green-Fed"	Rumen Material§	*Lacto-bacillus* spp. *	*Escheri-chia coli**	*Bacillus subtilis*†	Whole Egg Protein‡
Arginine	16·3	11·8	6·2	6·2-9·7	10·4	7·6	12·9
Histidine	4·8	6·2	2·4	2·6-4·1	3·3	2·5	3·6
Lysine	6·2	5·0	6·8	5·7-9·2	7·4	7·5	8·6
Threonine	2·9	2·6	2·9	2·4-3·6	2·8	2·6	3·6
Alanine	2·9	4·0	—	—	—	—	—
Phenylalanine	2·2	2·0	1·3	1·4-2·2	1·7	1·9	3·3
Leucine	4·5	4·0	3·3	3·4-5·0	5·0	5·1	6·1
Isoleucine	2·8	2·7	2·25	3·3-4·7	4·2	3·2	5·3
Valine	3·7	4·9	2·9	3·5-4·1	4·6	4·2	5·5
Tryptophane	1·4	1·2	0·5	0·3-0·5	0·6	0·5	1·3
Methionine	1·1	1·2	0·95	0·6-0·8	1·2	1·0	2·4
Tyrosine	2·5	1·9	—	—	1·4	1·5	2·2
Aspartic Acid	7·2	7·0	—	—	—	—	—
Glutamic Acid	11·5	12·1	—	5·8-6·9	—	—	—
Glycine	2·9	4·0	—	—	—	—	2·6

* Calculated from data of Camien, Salle, and Dunne (1945).
† Calculated from data of Stokes and Gunness (1946).
‡ Calculated from data given by Block and Bolling (1946).
§ Calculated from data of Loosli *et al.* (1949).

comparison with our own results. It should be noted, however, that in all cases no allowance has been made for hydrolytic losses so that the results for the basic amino acids and for tyrosine and methionine are almost certainly too low. Certain other differences should also be mentioned. Our figures were obtained by chromatography on protein preparations of the rumen bacteria. The results of Camien, Salle, and Dunne (1945), and of Stokes and Gunness (1946) were obtained by microbiological assay of hydrolysates of whole washed bacterial cells. The data of Loosli *et el.* (1949) for rumen material were also obtained by microbiological assay and are stated as being "minimal."

The two rumen bacterial samples exhibit, on the whole, very similar patterns of amino acid distribution. Of the essential amino acids the "dry-fed" sample

contains appreciably higher amounts of arginine and lysine and smaller amounts of histidine and valine. The methionine contents of the two samples, namely 1.1 per cent. "dry-fed" and 1.2 per cent. "green-fed," are of the same order as the values found for the same material by Reed, Moir, and Underwood (1949), who used the "differential-oxidation" method of Lugg (1938). The figures given by these workers, namely 1.2 per cent. methionine N for the "dry-fed" sample and 1.5 per cent. for the "green-fed" indicate, however, a greater difference between the two samples.

A comparison of the rumen bacteria values with those of other bacteria given in Table 1 reveals no outstanding differences. The levels of lysine, threonine, phenylalanine, leucine, and valine are very similar in all the organisms studied, whilst methionine differs only in the low values recorded for the *Lactobacillus* spp. Arginine, histidine, trypotophane, and glutamic acid are appreciably higher in the isolated rumen bacterial protein than in the proteins of the other materials recorded, and isoleucine is slightly lower. The data obtained from these mixed populations provide further support for the general conclusions reached by earlier workers that the total proteins of microorganisms do not differ markedly in amino acid composition and that this composition is not greatly influenced by the conditions of growth.

IV. Discussion

The finding from our own data and those of other workers that the bacterial protein, even when grown under widely varying conditions, is probably very similar in amino acid composition implies that rumen bacterial protein is relatively constant in composition for different conditions in the rumen. Since a varying but often high proportion of dietary protein and of non-protein N is converted in the rumen to bacterial protein and this protein becomes a major source of essential amino acids to the host animal it is of interest to examine its amino acid composition in relation to other proteins in the ruminant diet.

Whole egg protein can hardly be regarded as a normal source of N to the ruminant but it is of particular interest because it is almost perfectly utilizable for growth in the rat and is therefore regarded as possessing a practically ideal balance of essential amino acids for this purpose. Rumen bacterial protein, in comparison with this protein, is well supplied with arginine, histidine, valine, and tryptophane, is deficient in leucine, threonine, and phenylalanine, and is very deficient in methionine and isoleucine. In fact, the "limiting" amino acids, in the Mitchell and Block (1946) sense of that term, are almost equally methionine and isoleucine, as each of these has a percentage deficit of about -50.

A comparison with more common sources of protein taken from the data of Holmes (1953), using the "total per cent. deficit" of essential amino acids in each case as described by Mitchell and Block (1946), indicates that rumen bacterial protein is inferior to vetch and lupin seed protein, of similar value to linseed and meatmeal, slightly superior to pea seed, and markedly superior to subterranean clover seed protein. The marked superiority to subterranean clover seed protein is of particular interest because this protein is even more

severely deficient in isoleucine, methionine, and threonine than rumen bacterial protein itself, whereas vetch seed protein, for instance, is much better supplied with these three amino acids.

If it is assumed that a considerable proportion of dietary protein is converted to bacterial protein the comparisons just made suggest that, where the amino acid composition of rumen bacterial protein is inferior to that of a dietary protein, as with whole egg protein, the biological value (B.V.) of this dietary protein should be lower in the ruminant than in the rat. Support for this contention is given by the fact that Block and Mitchell (1946) report the figure of 96 for the B.V. of whole egg protein in the rat, compared with 86.7 obtained by Williams and Moir (1951) for this protein in growing lambs. Further, with linseed protein, in which the amino acid composition is, on our data and those of Holmes (1953), rather similar to that of rumen bacterial protein, the B.V. for growth in both the rat and the sheep are closely similar. Thus Block and Mitchell give the value of 78 for rats and Williams and Moir the value of 79.7 for lambs. Williams and Moir have also found no significant difference between the B.V. of linseed meal protein and subterranean clover seed protein in lambs, in spite of the much better essential amino acid make-up of the former (Holmes 1953). Moreover these workers obtained for urea a B.V. of 69 where this source of N constituted approximately half the dietary N of lambs, and Loosli *et al.* (1949) a B.V. of 46 where urea constituted the sole dietary source of N to lambs. Since urea supplies no essential amino acids it has obviously no biological value to rats. It is clear, therefore, that comparisons between the B.V. of dietary protein in ruminants and non-ruminants are of little value.

The utilization of dietary N in the ruminant depends upon the course of its metabolism within the rumen and is influenced by numerous factors such as its form and source, the level of intake, and immediate energy considerations (McDonald 1952; El-Shazly 1952; Synge 1952). Ingested N suffers a variable bacterial degradation to ammonia which may either be directly absorbed from the rumen (McDonald 1948*b*) and lost in whole or in part in the urine as urea or used as a source of N for protein synthesis by the ruminal bacteria where suitable carbohydrates are present. It is obvious that the ultimate value of any N source to the ruminant will be profoundly influenced by the degree to which one or other of these two processes of breakdown and synthesis predominate. Very little is yet known of the factors influencing either.

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VI. References

Beadles, J. R., Quisenberry, J. H., Nakamura, F. I., and Mitchell, H. H. (1933).—*J. Agric. Res.* **47**: 947.

Block, R. J., and Bolling, D. (1946).—"The Amino Acid Composition of Proteins and Foods." (C. C. Thomas, Springfield, Ill., U.S.A.)

BLOCK, R. J., and BOLLING, D. (1951).—"The Amino Acid Composition of Proteins and Foods." (C. C. Thomas, Springfield, Ill., U.S.A.)
BLOCK, R. J., and MITCHELL, H. H. (1946).—*Nutr. Abstr. Rev.* **16**: 249.
BOISSONNAS, R. A. (1950).—*Helv. Chim. Acta* **33**: 1966.
CAMIEN, M. N., SALLE, A. J., and DUNNE, M. S. (1945).—*Arch. Biochem.* **8**: 67.
CONSDEN, R., GORDON, A. H., and MARTIN, A. J. P. (1944).—*Biochem. J.* **38**: 224.
CONSDEN, R., GORDON, A. H., and MARTIN, A. J. P. (1947).—*Biochem. J.* **41**: 590.
EL-SHAZLY, K. (1952).—*Biochem. J.* **51**: 640.
FOWDEN, L. (1951).—*Biochem. J.* **48**: 327.
FOWDEN, L., and PENNY, J. R. (1950).—*Nature* **165**: 846.
HOLMES, P. (1953).—*Aust. J. Exp. Biol. Med. Sci.* (in press).
JOHANSON, R., MOIR, R. J., and UNDERWOOD, E. J. (1949).—*Nature* **163**: 101.
JOHNSON, B. C., HAMILTON, T. S., MITCHELL, H. H., and ROBINSON, W. B. (1942).—*J. Anim. Sci.* **1**: 236.
JOHNSON, B. C., HAMILTON, T. S., ROBINSON, W. B., and GAREY, J. C. (1944).—*J. Anim. Sci.* **3**: 287.
KIK, M. C. (1938).—Arkans. Agric. Exp. Sta. Bull. No. 352.
LOOSLI, J. K., WILLIAMS, H. H., THOMAS, W. E., FERRIS, F. H., and MAYNARD, L. A. (1949).—*Science* **110**: 144.
LUGG, J. W. H. (1938).—*Biochem. J.* **32**: 2114.
LUGG, J. W. H. (1939).—*Biochem. J.* **33**: 110.
LUGG, J. W. H. (1949).—*Advanc. Protein Chem.* **5**: 229.
LUGG, J. W. H., and WELLER, R. A. (1944).—*Aust. J. Exp. Biol. Med. Sci.* **22**: 149.
MCDONALD, I. W. (1948*a*).—*J. Physiol.* **107**: 21P.
MCDONALD, I. W. (1948*b*).—*Biochem. J.* **42**: 584.
MCDONALD, I. W. (1952).—*Biochem. J.* **51**: 86.
MCNAUGHT, M. L., SMITH, J. A. B., HENRY, K. M., and KON, S. K. (1950).—*Biochem. J.* **46**: 32.
MITCHELL, H. H., and BLOCK, R. J. (1946).—*J. Biol. Chem.* **163**: 599.
MOIR, R. J., and WILLIAMS, V. J. (1950).—*Aust. J. Sci. Res.* B **3**: 381.
MOORE, S., and STEIN, W. H. (1948).—*J. Biol. Chem.* **176**: 367.
PARTRIDGE, S. M. (1948).—*Biochem. J.* **42**: 238.
PHILLIPS, D. M. P. (1948).—*Nature* **167**: 53.
REED, F. M., MOIR, R. J., and UNDERWOOD, E. J. (1949).—*Aust. J. Sci. Res.* B **2**: 304.
STOKES, J. L., and GUNNESS, M. (1946).—*J. Bact.* **52**: 195.
SYNGE, R. L. M. (1952).—*Brit. J. Nutrit.* **6**: 100.
DE VERDIER, C. H., and AGREN, G. (1948).—*Acta Chem. Scand.* **2**: 783.
WILLIAMS, R. J., and KIRBY, H. (1948).—*Science* **107**: 481.
WILLIAMS, V. J., and MOIR, R. J. (1951).—*Aust. J. Sci. Res.* B **4**: 377.
WORK, E. (1949).—*Biochim. Biophys. Acta* **3**: 400.