Some Observations on the Nitrogen Metabolism of Rumen Proteolytic Bacteria

By A. R. ABOU AKKADA* AND T. H. BLACKBURN

The Rowett Research Institute, Bucksburn, Aberdeen

(Received 1 November 1962)

SUMMARY

Isolates from the rumens of sheep of presumptively identified *Bacteroides amylophilus*, *B. ruminicola*, species of *Bacteroides*, *Selenomonas*, *Butyrivibrio*, *Bacillus*, *Eubacterium*, *Clostridium* and Gram-positive cocci were found to be proteolytic. Some of these strains also had exopeptidase and amidase activity, but significant deaminase activity was rare. Most of the strains preferentially utilized ammonia in synthesizing cellular constituents in media containing preformed amino acids. Few of the strains had urease activity.

INTRODUCTION

In a survey of the proteolytic bacteria from the sheep rumen (Blackburn & Hobson, 1962) it was found that proteolytic activity was possessed by strains of many kinds of bacteria. Proteolytic bacteria were selected on the basis of their capacity to produce some visible change in casein agar roll tubes, and overall proteolytic activity in liquid cultures was measured by determining the decrease in trichloroacetic acid-precipitated protein. The cultures isolated were not stored for more detailed investigation partly because of the difficulty of keeping many of the bacteria viable over prolonged periods. For the present work proteolytic strains of bacteria were re-isolated to provide further confirmation that the previously described bacteria are found in the rumen at all times, and the nitrogen metabolism was investigated in more detail. Previously it was found that a well-reduced medium was needed for good growth of proteolytic bacteria, and for most isolations media containing cysteine + dithionite were used, although media with cysteine alone were used initially. In the present work both types of media were used. As before, media containing a number of carbohydrates were used, to ensure that as large a number as possible of bacterial types grew. Since, as previously noted, proteolytic activity in liquid medium did not always coincide with a visible change in a solid casein medium, representatives of all colonial types which grew in the primary media were tested for proteolysis in liquid media.

METHODS

Sheep. Isolations were made from five sheep; sheep 6 was fed on a partially-defined casein diet, sheep 190, 74, 93 and 95 on hay-concentrate diets (Blackburn & Hobson, 1962). Sheep 95 by special experimental procedure contained no protozoa (Eadie & Hobson, 1962).

* Present address: Faculty of Agriculture, University of Alexandria, Alexandria, Egypt.

Isolation and identification. The methods used were as previously described (Blackburn & Hobson, 1962), with the following modifications. Carbohydrates were added at 0.1 % concentration to discourage colonies from spreading. Isolation media 1D and 1c, 2D and 2c, 3D and 3c, 4D and 4c contained, respectively, no carbohydrate, carboxymethyl cellulose, glucose, and a mixture of xylose, maltose and cellobiose. Media designated D were reduced by cysteine + dithionite, media designated c were reduced by cysteine alone. Colony development was followed, any changes produced in the casein noted, and finally representatives of all colony types subcultured on agar slopes, a count being made of all similar colony types. The morphology of the organisms growing on the slopes was examined and the strains were inoculated into casein liquid medium to determine their proteolytic activity (Blackburn & Hobson, 1960*b*). Only proteolytic strains were examined further.

Nitrogen metabolism. This was studied in a basal medium to which various additions were made as indicated in Table 1. The media were incubated for 4 days before analysis.

Table 1. *Additions to the basal medium used in studying the nitrogen metabolism of proteolytic bacteria*

Additions and concentration	Media					
	C.Y. NH ₃	T.Y. NH ₃	T	T. NH ₃	T.A. NH ₃	T.Y.U.
Casein 0.5 %	+	—	—	—	—	—
Tryptose 0.5 %	—	+	+	+	+	+
Yeast extract 0.1 %	+	+	—	—	—	+
Urea 0.3 %	—	—	—	—	—	+
Asparagine 0.132 %	—	—	—	—	+	—
(NH ₄) ₂ SO ₄ 0.3 %	+	+	—	+	+	—

+ = The addition is present in the medium.

— = The addition is not present in the medium. Additions were at final concentrations as described above.

The basal medium contained per 100 ml.: salt solutions *a* and *b* (Blackburn & Hobson, 1962) minus ammonium sulphate, 15 ml. of each; centrifuged rumen fluid, 10 ml.; sodium bicarbonate 0.5 g.; L-cysteine-HCl, 0.05 g.; dithionite, 0.003 g.; pheno-safranine, 0.0001 g.; xylose, maltose and cellobiose, 0.1 g. of each.

Analytical methods. Uninoculated media were used as controls. A micro-Kjeldahl method (McKenzie & Wallace, 1954) was used to determine nitrogen in centrifuged bacterial deposits, and for trichloroacetic acid (TCA) soluble- and insoluble-nitrogen (Blackburn & Hobson, 1960*a*). Proteolytic activity was measured by decrease in TCA-insoluble nitrogen. Urease activity was measured by the production of ammonia from urea, amidase activity was measured by the production of ammonia from asparagine (Conway, 1957). Deaminative activity was measured by the production of ammonia in C.Y. NH₃, T.Y. NH₃ and T media (Table 1). A measure of the amino acid and peptide-nitrogen production in C.Y. NH₃ medium was obtained as follows. Samples (1.0 ml.) of the culture media were adjusted to pH 4.0 by adding citric acid (0.1 M) and diluted to 10 ml. by adding phosphate-citrate buffer (pH 4.0). After filtration through Whatman No. 42 paper, samples (0.1 ml.) of the filtrate were used for determining amino acid-nitrogen by the method of Lampson & Singher (1960) as modified by Abou Akkada & Howard (1962). Dipeptide nitrogen

was similarly determined on another sample of the culture adjusted to pH 5.5. Polypeptide-nitrogen was determined by mixing 1 ml. of the cell-free supernatant fluid of a centrifuged culture with 2 ml. 10 % (w/v) trichloroacetic acid, standing for 1 hr. and filtering through Whatman No. 42 paper. Samples (1.0 ml.) of the filtrate were mixed with 2N-NaOH, 1 ml., and biuret reagent, 8 ml., and the procedure of Layne (1957) followed. The qualitative and quantitative determinations of volatile fatty acids, except formic acid, were as previously described (Abou Akkada & Howard, 1960). Formic acid was detected by the ninhydrin method of Burness & King (1958). Quantitative determination of formic acid was carried out on a distillate of the culture medium by the colorimetric micro-method of Grant (1947). Succinic and lactic acids were identified by paper chromatography of the culture medium by a method similar to that of Stark, Goodban & Owens (1951).

RESULTS

One series of isolations was made from each sheep, 6, 190, 74, 93 and 95 and the runs were named B, C, D, E and F, respectively. The eight isolation media have been described (see page 462). The isolated bacteria were named according to the particular run, the medium and the dilution in which they appeared. Thus D1D6 would be a strain isolated in run D (sheep 74) from a medium containing no carbohydrate, reduced by cysteine + dithionite and occurring at 10^6 organisms/ml. rumen fluid. A further number, e.g. D1D63, would differentiate that strain from others isolated from the same dilution roll tube.

Media reduced by cysteine + dithionite were no more effective than those reduced by cysteine alone in producing a proteolytic flora. No particular carbohydrate source stimulated the growth of proteolytic bacteria but glucose appeared to have an inhibitory effect. Proteolytic colonies could easily be seen in the media containing no carbohydrate or carboxymethyl cellulose after incubation for 4 days. Probably no particular bacterial proteolytic type was associated exclusively with any sheep. Some colonies which produced no definite change or zone in the agar roll tubes produced marked hydrolysis of casein in liquid culture and vice versa. Proteolytic bacteria did not contribute to more than 10 % of the viable population on these media.

Representative proteolytic bacterial strains from each run were selected and these strains (32) were identified and their nitrogen nutrition examined. The properties of the bacteria were determined by using the same range of tests as did Blackburn & Hobson (1962) and they were classified in a similar manner. The products of fermentation which are helpful in classifying the bacteria are listed in Table 2.

Group 1. *Bacteroides*

(a) *Bacteroides amylophilus*. These seven strains (B3D71, B3D75, E4C6, E1D6, C1C6, C2C62, D1D61) were pleomorphic Gram-negative $1\ \mu$ coccoids, non-motile, and fermented dextrin, glycogen, starch and maltose.

(b) *Bacteroides ruminicola*. These three isolates (E2C7, E4D8, F2D7) were pleomorphic Gram-negative, non-motile rods 0.3 to $0.5 \times 1.0\ \mu$, and fermented a wide range of carbohydrates.

(c) *Bacteroides* sp. This strain (E4c6) was a Gram-negative small curved $0.4 \times 1.4 \mu$ pointed-end rod; it produced butyric acid from glucose.

Group 2. *Lachnospira*. This single isolate, B2D8, was a Gram-negative, long ($0.4 \times 2.5 \mu$), slightly curved, rod occurring in chains. It fermented only glycerol, glucose, mannose, maltose, sucrose, trehalose, carboxymethyl cellulose and cellobiose. The original colony produced no visible change in the casein of the roll tube.

Table 2. *Fermentation products in fluid medium after 4 days incubation at 38° C*

Isolate	Sugar fermented	Total† VFA m-equi- valents/ 100 ml.	Succinic acid	Lactic acid	% of total VFA				Final pH	Group
					Formic	Acetic	Pro- pionic	Butyric		
B3D71	Maltose	6.5	+	—	25	69	7	4	6.3	1
B3D75	Maltose	6.9	+	—	25	58	7	5	6.4	
E4c6	Maltose	3.2	+	—	22	69	8	4	5.1	
E1D6	Maltose	*	+	—	30	60	7	4	*	
C1c6	Maltose	2.9	+	—	43	44	9	4	7.1	
C2c62	Maltose	*	*	*	*	*	*	*	*	
D1D61	Maltose	3.7	+	—	30	60	7	4	5.3	
E2C7	Glucose	1.3	+	+	30	50	17	2	5.2	
E4D8	Glucose	1.8	+	+	0	84	11	2	5.8	
F2D7	Glucose	1.2	+	+	30	52	14	4	5.6	
E4c6	Glucose	5.3	+	—	30	21	4	49	5.4	2
B2D8	Maltose	7.3	+	—	30	58	7	5	6.3	
D1D4	Glucose	2.0	+	—	22	16	8	47	*	3
D4D6	Glucose	4.3	+	+	27	27	4	28	*	
F1D6	Glucose	2.5	+	+	32	28	6	46	5.6	
F2D6	Glucose	3.4	+	+	0	30	4	65	5.5	
F3D61	Glucose	3.1	+	+	20	18	6	52	5.3	
F4c6	Glucose	2.1	+	+	0	6	48	42	4.5	
E2c63	Glucose	1.8	—	+	10	57	9	25	4.6	4
E2c65	Glucose	1.6	—	+	8	53	10	28	4.4	
E2c62	Glucose	1.7	—	+	24	35	6	24	4.4	
F2c61	Glucose	1.4	—	+	0	78	20	5	5.0	
F4c6	Glucose	1.9	+	+	12	51	34	3	4.5	
F4D71	Glucose	2.1	—	+	*	46	27	9	4.2	
F3D6	Glucose	1.8	—	+	27	50	18	5	5.0	5
C2c6	Maltose	6.3	—	—	20	57	8	22	6.1	
E2c64	Glucose	1.9	—	+	24	19	7	50	4.4	
D2D6	Glucose	1.0	+	+	51	38	7	4	*	
E1c7	Glucose	1.5	—	+	8	53	20	3	5.8	
F4D7	Glucose	1.2	—	+	0	58	13	19	4.2	6
F3c6	Glucose	4.3	—	+	22	25	6	28	4.8	
F1D8	Glucose	1.3	—	+	30	49	13	9	4.7	

* Not determined.

— No production.

+ Production.

† VFH = volatile fatty acids.

Group 3. *Butyrivibrio*. These six isolates (D1D4, D4D6, F1D6, F2D6, F3D61, F4c6) were Gram-negative curved rods 0.3 to 0.5×1 to 2.5μ , motile (except D1D4) by a single polar flagellum, and fermented a wide variety of carbohydrates.

Group 4. *Selenomonas ruminantium*. These eight isolates (E2c63, E2c65, E2c62, F2c61, F4c6, F4D71, F3D6, c2c6) were large Gram-negative crescents 0.4 to

1.2 × 2.0 to 4.0 μ , motile with flagellation similar to that described by Hobson & Mann (1961). Isolate r2c61 could be classified as *Selenomonas ruminantium* var. *lactyliticus*.

Group 5. Gram-positive or Gram-variable long rods

(a) Eubacterium. Isolate e2c64 was a non-motile, non-sporing, 0.6 × 2.3 μ rod in fairly long chains, and fermented arabinose, dextrin, fructose, glucose, galactose, glycogen, inulin, lactose, mannitol, mannose, maltose, sucrose and starch.

(b) Bacillus. Organism d2d6 was a Gram-positive, non-sporing 0.9 × 2 μ facultative anaerobic rod; it fermented a moderate range of carbohydrates.

(c) Clostridium. The isolate e1c7 was a Gram-variable non-motile rod 0.4 × 2.4 μ with a swollen medial spore, and fermented a moderate range of carbohydrates.

Group 6. Gram-positive cocci

The isolates f4d7, f3c6, f1d8, all 1.0 μ diameter cocci, were included in this group; f1d8 was facultatively anaerobic.

Peptidase activity

The end products of casein degradation by proteolytic bacteria were mainly amino acids and polypeptides (Table 3). No dipeptides were ever observed. Endopeptidase or proteinase activity was indicated by the percentage breakdown in casein after growth in casein liquid medium, exopeptidase activity by the ratio of amino acid-nitrogen to total amino acid + polypeptide-nitrogen (Table 3). Proteolytic activity varied within the presumptively identified groups as did exopeptidase activity and there was no correlation between the two except that the *Bacteroides amylophilus* strains had mostly high proteolytic activity and low exopeptidase activity while the *B. ruminicola* group had consistently high exopeptidase activity. No generalizations could be made regarding the *Butyrivibrio* and *Selenomonas* groups.

Ammonia production

The results of experiments to test ammonia production or utilization are tabulated (Table 4) in the form of increase or decrease in ammonia concentration over the control after incubation. The amount of bacterial growth was estimated only for the C.Y.NH₃ medium. In some cases this gave a very low value because of autolysis of the organisms, and sometimes the value was too high because denatured casein was sedimented with the centrifuge-deposit of organisms, this often being associated with amidase activity (Table 4). Probably the average bacterial nitrogen content should have been 1.0 to 2.0 μ equivalents nitrogen/ml. culture fluid. There was very little evidence of ammonia production from casein in the medium C.Y.NH₃; in general there was an uptake of ammonia corresponding roughly with the bacterial nitrogen. Deamination could result in an increase in ammonia in all the media. Media C.Y.NH₃, T.Y.NH₃ and T.A.NH₃ contained 5.0, 0.5 and 10.0 μ equivalents amide-nitrogen/ml., respectively, and thus amidase activity could also release ammonia in these media. Urease activity would be detected in T.Y.U. medium. The T.Y.NH₃ medium differed from the C.Y.NH₃ in containing a trypsin-hydrolysed casein, tryptose. In T.Y.NH₃ medium as in C.Y.NH₃ medium there was generally a net uptake of ammonia and there was no evidence that tryptose was a better

substrate for deaminases than was casein. A comparison between media T and T.NH₃ showed that in many cases the higher initial ammonia level resulted in decreased net production of ammonia. This was not true of the *Butyrivibrio* group 3. A comparison between T.NH₃ and T.A.NH₃ showed the amount of amidase activity. This property was found in all three strains of *Bacteroides ruminicola* group 1, and occurred

Table 3. *The products of casein hydrolysis after 4 days of incubation at 39° C*

Isolate	Casein hydrolysed (%)	Amino acid-nitrogen % total casein-nitrogen	Polypeptide-nitrogen as % total casein-nitrogen	Amino acid-nitrogen as % non-protein-nitrogen	Group
B3D71	86	15	61	20	1
B3D75	86	14	65	18	
E4C6	30	5	26	15	
E1D6	35	11	26	34	
C1C6	90	16	68	20	
C2C62	94	31	54	37	
D1D61	87	19	59	24	
E2C7	28	8	10	44	
E4D8	97	64	17	79	
F2D7	46	23	26	45	
E4C6	28	9	18	35	2
B2D8	33	5	29	14	
D1D4	55	23	29	44	
D4D6	16	5	13	28	3
F1D6	81	19	58	25	
F2D6	66	7	29	18	
F3D61	43	18	26	42	
F4C6	87	23	66	26	
E2C63	93	36	57	38	4
E2C65	49	7	39	16	
E2C62	40	6	35	15	
F2C61	55	15	33	31	
F4C6	12	2	8	16	
F4D71	96	17	71	20	
F3D6	21	4	14	20	
C2C6	34	8	27	23	5
E2C64	47	6	42	13	
D2D6	26	6	17	26	
E1C7	90	61	27	69	6
F4D7	93	15	71	18	
F3C6	12	2	14	11	
F1D8	79	10	64	14	

The initial casein concentration was 50 μ -equivalents nitrogen/ml. of medium C.Y. NH₃, the composition of which is in Table 1.

in *Butyrivibrio* group 3 (strains F2D6, F3D61, F4C6); in *Selenomonas* group 4 (strains E2C63, E2C65, F4C6, F4D71, F3D6) and in all strains in groups 5 and 6. The strains E4D8, F2D7 of *B. ruminicola*, *Butyrivibrio* F3D61, *Selenomonas* strains F3D6 and possibly F2C61, F4C6, F4D71, all strains in group 5, strain F4D7 in group 6 were considered to show some degree of deaminative activity. No significant urease activity was shown by any of these bacteria except possibly F4D7 and F2D6, and even there only a small amount of the substrate was attacked.

Table 4. *Production of ammonia from various substrates after 4 days of incubation at 39°*

The results are expressed in μ -equivalents of nitrogen per ml. of culture fluid. The initial concentrations of ammonia-nitrogen in μ -equivalents per ml. of uninoculated C.Y. NH₃, T.Y. NH₃, T, T.A. NH₃, T. NH₃ and T.Y.U. were 15.0, 17.0, 1.5, 15.3, 15.3 and 4.9 approximately, depending on the batch of medium. Media composition in Table 1.

Isolate	Bacterial nitrogen	Increase in ammonia nitrogen						Group
	C.Y. NH ₃	C.Y. NH ₃	T.Y. NH ₃	T	T. NH ₃	T.A. NH ₃	T.Y.U.	
B3D71	0.00	-3.72	0.48	-0.54	*	*	*	1
B3D75	0.00	-0.48	-0.46	-0.07	*	*	*	
E4C6	0.60	*	-2.86	-0.86	-1.64	0.71	1.21	
E1D6	0.90	*	-0.11	0.35	-1.19	0.71	-1.07	
C1C6	0.71	-1.18	-0.34	-0.15	*	*	*	
C2C62	*	-0.34	-1.43	0.33	-0.14	*	0.49	
D1D61	0.00	-1.64	-2.86	-0.50	-2.00	-1.64	-3.00	
E2C7	0.63	-1.07	-1.43	0.63	-0.93	3.14	0.50	
E4D8	6.45	13.32	3.57	6.78	-3.00	15.71	3.58	
F2D7	7.85	4.28	-0.71	1.47	-0.71	3.57	4.64	
E4C6	4.67	*	-1.46	-0.64	-0.53	*	-1.26	2
B2D8	1.93	-2.00	0.19	-0.59	*	*	*	
D1D4	1.60	-6.72	-1.00	-1.36	-1.93	-0.87	-0.60	3
D4D6	1.30	-0.38	-0.27	-0.28	0.43	-0.67	-0.20	
F1D6	2.00	-1.64	-3.66	-0.07	0.00	0.56	0.18	
F2D6	4.07	-1.93	-0.27	0.09	0.86	16.16	6.56	
F3D61	2.71	2.86	3.36	0.09	0.93	15.85	2.71	
F4C6	5.00	0.24	-0.48	1.07	2.50	16.43	1.79	
E2C63	10.91	*	0.91	0.36	-1.53	8.71	0.50	4
E2C65	9.14	*	-0.34	1.00	-1.33	9.30	0.15	
E2C62	7.14	*	0.48	0.50	-0.86	0.71	1.09	
F2C61	1.93	-1.93	-3.21	1.50	1.32	1.19	0.46	
F4C6	2.50	-2.64	-3.24	1.14	3.31	15.30	0.51	
F4D71	1.93	-2.00	-4.96	1.29	1.50	13.83	1.79	
F3D6	0.23	1.14	0.00	5.07	0.71	5.79	1.50	5
C2C6	3.07	-0.13	-1.00	-0.93	-0.26	0.81	-0.11	
E2C64	7.85	*	-0.26	4.14	-1.00	5.71	-2.43	
D2D6	8.14	1.50	2.07	0.90	0.19	3.36	-0.02	
E1C7	4.72	13.36	4.36	13.14	2.71	19.57	3.14	
F4D7	2.50	0.00	-3.86	1.72	3.78	17.14	8.72	6
F3C6	0.71	-1.29	-0.71	1.00	-2.00	4.50	2.07	
F1D8	1.20	-0.68	0.33	1.14	-2.00	1.79	*	

* Not determined.

DISCUSSION

Species of *Bacteroides*, *Selenomonas* and *Butyrivibrio* have again been found associated with protein hydrolysis in the sheep rumen. This is in keeping with the observations of Blackburn & Hobson (1962), but none of the large Gram-negative butyric acid-forming rods (group 5, Blackburn & Hobson, 1962) was isolated. Proteolytic bacteria did not constitute a major portion of the strains which appeared on the isolation media, thus differing from the observations of Fulghum, King & Moore (1958) with cattle who found 30 % of their total count to be proteolytic. As reported previously (Blackburn & Hobson, 1962) no particular carbohydrate encouraged the

growth of all proteolytic bacteria but maltose was effective in culturing *Bacteroides amylophilus*, a particularly active protease producer. The total absence of carbohydrate or the presence of a less readily attacked carbohydrate such as carboxymethyl cellulose, resulted in the isolation of strains that might otherwise have been overgrown. Occasionally strains (e.g. c1 c6, c2 c62, r2 c61) which had produced no change in the casein of the isolation medium, were found to be actively proteolytic in liquid culture. Generally, strains which produced a clear zone in the casein of the isolation medium subsequently produced hydrolysis of casein in liquid culture, but exceptions to this were noted, especially where the zone was not well defined.

A pure proteolytic enzyme such as crystalline trypsin will liberate little or no free amino nitrogen (Van Slyke, Dillon, MacFadyen & Hamilton, 1941). The present observations indicated that most of the strains had considerable exopeptidase activity, in addition to their proteolytic activity, which resulted in the liberation of free amino acids. This would be in accord with the results of Annison (1956) who found a rapid increase in amino acids and peptides in the rumen after feeding sheep a casein-containing diet. Considerable amidase activity was found in the present strains. The deamidation of casein by mixed rumen micro-organisms and by protozoa has been reported by Warner (1955) and Abou Akkada & Howard (1962). This amidase activity would explain the ability of propionamide to replace protein in ruminant diets (Hale, 1956).

Few of the isolates had any deaminase activity except in the *Bacteroides rumini-cola* and *Selenomonas* groups; others such as the *Clostridium* and Gram-positive cocci are probably of less significance in the total rumen population under normal conditions. *B. ruminicola* and selenomonads isolated from the bovine rumen have been reported by Bladen, Bryant & Doetsch (1961) to have deaminase activity. Many of the species reported in the present paper and by Blackburn & Hobson (1962), which have been isolated for the first time from the sheep rumen, are similar to those reported in the rumen of cattle (Bryant, 1959) and their nitrogen metabolism is similar. In keeping with the observations of Bladen *et al.* (1961), many of the bacteria studied appeared to utilize ammonia as the main source of nitrogen even in the presence of pre-formed amino acids and although they were hydrolysing casein.

No significant urease activity was demonstrated among the present isolates. The possession of urease has not yet been associated with any of the more common rumen micro-organisms even though urea is rapidly hydrolysed in the rumen.

The authors would like to thank Mr J. McIntosh and Mr R. Summers for skilled technical assistance and Dr P. N. Hobson for his helpful advice and criticism during this work.

REFERENCES

- ABOU AKKADA, A. R. & HOWARD, B. H. (1960). The biochemistry of rumen protozoa. 3. The carbohydrate metabolism of Entodinium. *Biochem. J.* **76**, 445.
ABOU AKKADA, A. R. & HOWARD, B. H. (1962). The biochemistry of rumen protozoa. 5. The nitrogen metabolism of Entodinium. *Biochem. J.* **82**, 313.
ANNISON, E. F. (1956). Nitrogen metabolism in the sheep. Protein digestion in the rumen. *Biochem. J.* **64**, 705.
BLACKBURN, T. H. & HOBSON, P. N. (1960*a*). Proteolysis in the sheep rumen by whole and fractionated rumen contents. *J. gen. Microbiol.* **22**, 272.

- BLACKBURN, T. H. & HOBSON, P. N. (1960*b*). Isolation of proteolytic bacteria from the sheep rumen. *J. gen. Microbiol.* **22**, 282.
- BLACKBURN, T. H. & HOBSON, P. N. (1962). Further studies on the isolation of proteolytic bacteria from the sheep rumen. *J. gen. Microbiol.* **29**, 69.
- BLADEN, H. A., BRYANT, M. P. & DOETSCH, R. N. (1961). A study of bacterial species from the rumen which produce ammonia from protein hydrolyzate. *Appl. Microbiol.* **9**, 175.
- BRYANT, M. P. (1959). Bacterial species of the rumen. *Bact. Rev.* **23**, 125.
- BURNES, A. T. & KING, H. K. (1958). Detection of fatty acids on paper chromatograms by means of ninhydrin. *Biochem. J.* **68**, 32*r*.
- CONWAY, E. J. (1957). *Microdiffusion Analysis and Volumetric Error*, 4th ed. London: Crosby Lockwood and Son, Ltd.
- EADIE, J. M. & HOBSON, P. N. (1962). Effect of the presence or absence of rumen ciliate protozoa on the total rumen bacterial count in lambs. *Nature, Lond.* **193**, 503.
- FULGHUM, R. S., KING, K. W. & MOORE, W. E. C. (1958). Development and comparison of media for the isolation of proteolytic bacteria from the rumen. *Bact. Proc.* 1958, 22.
- GRANT, W. M. (1947). Colorimetric micro-method for determination of formic acid. *Analyt. Chem.* **19**, 206.
- HOBSON, P. N. & MANN, S. O. (1961). The isolation of glycerol-fermenting and lipolytic bacteria from the rumen of sheep. *J. gen. Microbiol.* **25**, 227.
- HALE, W. H. (1956). Rumen metabolism of non-protein nitrogen. *J. Agric. Fd Chem.* **4**, 948.
- LAMPSON, G. P. & SINGHER, H. O. (1960). Colorimetric determination of amino acids in presence of a peptide. *Proc. Soc. exp. Biol., N.Y.* **103**, 368.
- LAYNE, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. *Methods in Enzymology*, vol. 3, New York: Academic Press, Inc.
- MCKENZIE, H. A. & WALLACE, H. S. (1954). The Kjeldahl determination of nitrogen. A critical study of digestion conditions—temperature, catalyst and oxidizing agent. *Aust. J. Chem.* **7**, 55.
- STARK, J. B., GOODBAN, A. E. & OWENS, H. S. (1951). Paper chromatography of organic acids. *Analyt. Chem.* **23**, 413.
- VAN SLYKE, D. D., DILLON, R. T., MACFADYEN, D. A. & HAMILTON, P. (1941). Gasometric determination of carboxyl groups in free amino acids. *J. biol. chem.* **141**, 627.
- WARNER, A. C. I. (1955). Ph.D. Dissertation, Aberdeen University. Some aspects of the nitrogen metabolism of the micro-organisms of the rumen with special reference to proteolysis.