the disulphide bond in the wool at all until considerable degradation has occurred.

Simple cystine polypeptides of varying compositions are now being synthesized to determine in which stereochemical arrangement if any the cystine disulphide bond cannot be oxidized by hypochlorite and permanganate.

SUMMARY

1. A study has been made of the reaction of the disulphide bond in wool with different oxidizing agents.

2. Chlorine, hypochlorous acid and peracetic acid are capable of oxidizing all the cystine in wool, whereas acid permanganate and alkaline hypo-

chlorite are only capable of oxidizing 25% of the cystine present. The cystine in wool may therefore be divided into two fractions, one of which is oxidized by the latter two reagents, and the other not.

3. The difference in reactivity of the two cystine fractions cannot be ascribed to morphological causes since the cystine of physically modified wools and powdered horn shows the same division.

4. The two fractions are not related to the Phillips (1946) cystine fractions. Possible explanations for the differences in reactivity are discussed.

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The Utilization of Non-protein Nitrogen in the Bovine Rumen*

5. THE ISOLATION AND NUTRITIVE VALUE OF A PREPARATION OF DRIED RUMEN BACTERIA

BY M. L. McNAUGHT AND J. A. B. SMITH
The Hannah Dairy Research Institute, Kirkhill, Ayr

AND K. M. HENRY AND S. K. KON
National Institute for Research in Dairying, University of Reading

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Pearson & Smith (1943) showed that when the liquid contents of the bovine rumen were incubated *in vitro* at 39° in the presence of a soluble carbohydrate such as starch or maltose, the non-protein nitrogen (N.P.N.) content of the liquid decreased markedly

* Previous papers in this series were published under the general title 'The Utilization of Urea in the Bovine Rumen'; see Smith & Baker (1944).

within 4 hr., suggesting that significant amounts of protein were formed. Subsequent work by Smith & Baker (1944) confirmed this suggestion and showed that the formation of protein could be attributed to the growth and multiplication of the bacteria which abound in the rumen liquid. In growing and multiplying, many of these bacteria not only synthesized protein, but were found also to build up and store a

starch-like polysaccharide. Smith & Baker succeeded in making small preparations of dried bacteria before and after incubation, but before the assumption could be made that the value of the non-protein nitrogenous substances of feeding-stuffs can be attributed to the formation of bacterial protein in the rumen, it was obviously necessary to make a preparation of the mixed bacteria which would be sufficiently large for its digestibility and biological value to be determined. The present paper describes the preparation and analysis at the Hannah Institute of over 450 g. of the material, and the determination of its digestibility and biological value in experiments with rats at the National Institute for Research in Dairying.

EXPERIMENTAL

The preparation of a sample of dried rumen bacteria. It was realized at the outset that in order to obtain about 400 g. of the dried bacteria, free from any significant amount of contamination with feeding-stuff residues from the rumen, very large volumes of rumen liquid would have to be dealt with, and steps would have to be taken to get the maximum yield possible from this liquid. There were several possibilities. The fistula animal which was used to obtain the rumen contents could either be kept at grass or it could be stall-fed; the bacteria could be separated from the rumen liquid either immediately the liquid was removed from the rumen or after incubation *in vitro* with added carbohydrate. In preliminary tests it was found that by incubating the rumen liquid with maltose before isolating the bacteria, the weight of dried bacteria obtained was increased by nearly 50%, and the amount of bacterial protein by 34%. It was decided, therefore, to incubate the rumen liquid before making the bacterial preparation. In the preliminary tests it was found that when the animal was grazing, higher yields of the bacterial preparation could be obtained than when it was stall-fed, but owing to the length of time which would obviously be necessary to complete the work, it was not practicable to keep the animal at pasture for the duration of the experiment. It was, therefore, decided to maintain the animal on indoor rations, and throughout these particular experiments it was fed a diet consisting of 16 lb. hay, 2 lb. bean meal, 2 lb. oats, 2 lb. dried grass, 2 lb. beet pulp and 15 lb. cabbage per day.

Over 400 g. of the preparation of dried bacteria were made in small batches over a period of 3 or 4 months. In a typical experiment 3 l. of rumen contents were removed from the rumen between 1 and 2 hr. after the animal had been fed. The contents were strained through muslin and cleared of protozoa and finely divided vegetable material by centrifugation for 5 min. at 2000 rev./min. ($800 \times g$). Maltose (1%) and urea (0.05%) were then added to the liquid and a sample taken for pH and N.P.N. determinations. The urea was added to ensure that there was ample N.P.N. present. The main bulk of the liquid was incubated for about 4 hr. at 39°. At the end of this time a further small sample was taken for the estimation of pH and N.P.N., and the remainder measured and 'clarified' by passing it through a Sharples super centrifuge. The speed of the centrifuge was approximately 24,000 rev./min., thereby giving a force of about $14,500 \times g$ on the surface of the rotating bowl, and the

liquid flowed through the centrifuge at the rate of 3 l./hr.

As the centrifuge slowed down, about 300 ml. of liquid rich in the smaller rumen bacteria flowed from the rotating bowl and were collected separately, while the bulk of the bacterial sediment adhered to the sides of the bowl as a thick slime. The liquid which drained off and the slime were placed immediately in ethanol, and the mixture was ground in a mortar. Shaking was not sufficient to disperse the bacteria. The bacterial suspension was then centrifuged and washed twice with ethanol and twice with ether. The residue was transferred to Petri dishes and the solvents allowed to evaporate, leaving a fine greyish white powder, which, when examined under the microscope, was found to consist mainly of bacteria and bacterial debris. The preparation from each batch of rumen liquid was stored in a refrigerator until the final batch had been dealt with. The preparations were then thoroughly mixed and the whole passed through a 90-gauge wire sieve. A total of 130 l. of rumen liquid treated in this way in fifty-seven incubations produced 461 g. of a dried bacterial preparation, a yield of 3.5 g./l.

Analysis of the bacterial preparation. The bacterial preparation was analysed by the generally accepted methods: N by Kjeldahl, total carbohydrate by the gravimetric Cu_2O method after hydrolysis, lipid by saponification followed by ether extraction, P by the colorimetric procedure of Fiske & Subbarow (1925) and Ca by the method of McCrudden (1911-12). In estimating total carbohydrate, allowance was made for the fact that on hydrolysis the furfural process for pentoses showed the presence of 4.1% pentose in the product. In the unhydrolysed product no free-reducing sugars were present. The preparation as analysed contained 7.5% moisture.

The analytical results calculated on a moisture-free basis are shown in Table 1 and are compared with figures given by Braude (1942) for dried yeast, and with typical figures for linseed cake. It will be seen from Table 1 that the protein and ash contents

Table 1. *Composition of the dried bacterial preparation compared with published values for dried fodder yeast and for linseed cake, a typical concentrate feeding stuff*

(The sample of dried bacteria contained 1.35% P and 0.29% Ca; the carbohydrates included pentose equivalent to 4.4% of the dried preparation. All percentage values in the table are calculated on a dry-weight basis.)

Component	Dried rumen bacteria (%)	Fodder yeast (Braude, 1942) (%)	Linseed cake (Woodman, 1945) (%)
Crude protein ($N \times 6.25$)	44.4	52.6	33.2
Carbohydrate other than 'fibre'	40.3	36.1	40.0
Lipid matter	3.1	1.1	10.7
'Crude fibre'	0.3	0.9	10.2
Ash	7.1	9.3	5.9
Total	95.2*	100.0	100.0

* Several attempts have been made to identify the remaining 4.8%, so far without success.

of the bacteria lay between those of the dried yeast and of the linseed cake, and that the carbohydrate content was much the same for all three products. The presence of so much phosphorus and pentose suggested that the preparation contained a considerable amount of nucleic acid. It is hoped that it will be possible to make a thorough investigation of the nature of these nucleic acids in the near future.

The proportion of the bacterial protein synthesized during incubation. In each of the fifty-seven incubations necessary to make 461 g. of a bacterial preparation with 7.5% moisture, records were kept of the decrease which occurred in the N.P.N. content of the liquid during the incubation period. It was calculated that in the whole experiment the total amount of N.P.N. converted to protein was 12.6 g., representing 78.8 g. protein, whereas the total amount of protein in the 461 g. of preparation was 189.5 g. It follows, therefore, that about 58% of the bacterial protein was present originally when the rumen liquid was taken from the rumen and that about 42% was synthesized during incubation.

The purity of the bacterial preparation. Before the nutritive value of the bacterial preparation was determined, it was important to know whether the material was contaminated to any significant extent with feeding-stuffs or with protein derived from them. Microscopical examination had already shown that any contamination which may have existed was very slight. The following considerations also indicated that the preparation consisted mainly of bacteria and bacterial debris. (a) The 'fibre' content of the preparation estimated by the method recommended by the Association of Official Agricultural Chemists (1945) was 0.3% (Table 1). Since the overall fibre content of the diet fed in this experiment would be about 14% the amount of contamination must have been very slight—perhaps as low as 2 or 3% of the total. It will be seen from Table 1 that dried fodder yeast may contain 0.9% 'fibre' which cannot possibly be contamination from feeding-stuffs so that the 0.3% recorded for the bacterial preparation may also not have arisen from this source. The contamination may therefore be even less than the 'fibre' content might suggest. (b) The possibility that the bacterial preparation might contain protein derived directly from feeding-stuffs was also investigated. An experiment was first made in which various feeding-stuffs were extracted with water at 37° for 2.5 hr. The feeding-stuffs were removed by the centrifuging procedure normally employed in this work to remove vegetable matter from the rumen liquid before incubation, and the amount of N.P.N. and protein nitrogen remaining in the supernatant layer was estimated. It was found that the only constituent of the diet from which protein was likely to be extracted was beans. This protein was, however, almost entirely soluble in dilute

Table 2. Individual and mean nitrogen metabolism data of twelve rats for 8-day periods showing the biological value and true digestibility of the bacterial protein

Rat no.	Initial low egg-nitrogen period						Experimental period						Final low egg-nitrogen period					
	Weight of rat (g.)			Weight of rat (g.)			Weight of rat (g.)			Weight of rat (g.)			Weight of rat (g.)			Weight of rat (g.)		
	Initial	Final	Diet intake (g.)	Faecal N (mg.)	Urinary N (mg.)	N intake (mg.)	Faecal N (mg.)	Urinary N (mg.)	Bio-logical value	True digestibility	Initial	Final	Diet intake (g.)	Faecal N (mg.)	Urinary N (mg.)	Initial	Final	Diet intake (g.)
1	54	56	34.72	85.3	70.9	617.0	262.0	120.8	88.9	74.7	74	80	46.10	91.9	68.7	74	80	46.10
2	55	59	40.71	91.6	76.0	541.1	226.7	117.7	90.5	75.2	70	76	42.13	92.4	77.4	70	76	42.13
3	58	60	39.29	88.2	78.9	642.8	274.7	139.6	87.6	73.7	78	89	55.18	112.0	84.1	78	89	55.18
4	56	58	33.60	72.5	75.3	626.7	270.1	144.4	86.0	73.8	70	77	44.04	98.0	86.4	70	77	44.04
5	56	62	47.59	104.0	78.2	681.9	296.7	145.2	88.4	72.4	77	87	57.11	111.3	99.9	77	87	57.11
6	55	62	44.48	88.4	76.1	681.9	288.8	130.3	89.9	72.7	80	89	58.35	112.8	82.6	80	89	58.35
7	56	59	37.45	79.7	75.8	668.8	305.2	178.8	81.1	71.4	72	75	44.43	101.3	99.0	72	75	44.43
8	49	56	48.63	97.9	76.2	675.4	319.3	149.2	88.1	69.2	70	72	40.96	92.9	106.3	70	72	40.96
9	46	51	42.81	74.9	79.9	675.4	273.2	131.7	90.6	74.7	70	78	58.70	128.2	84.2	70	78	58.70
10	48	53	38.77	81.1	73.0	675.6	280.6	130.5	89.6	74.3	73	73	48.99	98.7	83.7	73	73	48.99
11	54	54	25.88	57.8	78.1	589.2	257.1	126.0	87.2	72.6	72	80	49.24	97.4	67.7	72	80	49.24
12	54	58	42.89	91.0	69.9	681.9	293.7	121.7	91.1	73.9	78	82	51.85	119.0	80.5	78	82	51.85
									Mean	88.3								
									S.E.M.	±0.78								
										±0.56								

salt solution which makes it unlikely that it would be centrifuged from the rumen liquid during the preparation of the dried bacteria. However, to test the matter further, 5 g. of a mixture consisting of equal parts of bean meal, dried grass and bruised oats was incubated with 250 ml. rumen liquid at 37° for 1 hr., and then centrifuged to remove the feeding-stuff residues, a sample of the same rumen liquid being treated in the same way but without the addition of the feeding-stuff mixture. It was found that there was no significant difference in the level of N.P.N. and protein nitrogen in the resulting liquids, indicating that the bacterial preparation used in the present work for the determination of nutritive value was not likely to contain any significant amount of protein derived directly from feeding-stuffs.

The nutritive value of the protein of the bacterial preparation. The method adopted was essentially that of Mitchell (1923-4a) and Mitchell & Carman (1926), the details of which have been described by Henry, Kon & Watson (1937). The bacterial preparation was fed to twelve weanling female rats in a diet of which it formed 18.1 % of the dry weight, the remaining constituents being cane sugar 15.0, potato starch 10.0, rice starch 42.9, margarine fat 10.0 and salts (de Loureiro, 1931) 4.0 %. (The salt mixture was the same as that described by Campbell, Olley & Blewett (1949) except that it contained no zinc sulphate.) The diet, therefore, contained 8 % protein, all of which was supplied by the bacterial preparation. As fed, the diet contained 9.8 % moisture. Metabolic faecal nitrogen and endogenous urinary nitrogen were estimated during periods of 6 days before and after the experimental period which was also of 6 days. The results are shown in Table 2. The mean biological value for the twelve rats was 88.3 with a standard error of the mean of ± 0.78 , and the mean true digestibility 73.2 ± 0.56 . Two of the rats (nos. 7 and 8) showed a slight tendency to diarrhoea during the experimental feeding period, but this was rapidly overcome by slightly reducing the food intake.

DISCUSSION

Probably the earliest attempt to investigate the nutritive value of rumen bacteria was made by Müller (1906), who inoculated culture medium with rumen bacteria, and after allowing growth to proceed for 3 days, precipitated the mixture with ammonium sulphate, washed the precipitate with ethanol, and fed a bitch with the dried product. He found that nitrogen retention on the ration containing the bacterial protein was similar to that on rations containing albumin and casein. More recently, Usuelli & Fiorini (1938) made preparations of rumen bacteria, of rumen protozoa and of residues of feeding-stuffs from the rumen, and gave them as supplements to a basal diet in growth experiments

with chickens. The basal diet supplemented with the residues of feeding-stuffs was not significantly better in promoting growth than the basal diet alone, but the bacterial and protozoan preparations brought about more rapid growth. A further endeavour was made to determine the nutritive value of the bacterial protein of the rumen by Johnson, Hamilton, Robinson & Garey (1944), who made dried preparations of rumen bacteria, of protozoa and also of an organism which they isolated in pure culture from the mixed rumen flora. Unfortunately only about 80 g. of each preparation were available, with the result that only two or three rats could be used in the tests, and the results obtained for the individual rats varied greatly. Thus the three individual results for the biological value of the protein in the mixed rumen preparation were 56, 70 and 73. Moreover, the mixed bacterial preparation had a 'fibre' content as high as 1.4 %, so that it may have been seriously contaminated with feeding-stuff residues.

In the present work the biological value was found to be 88.2, which is high and compares favourably with that of dried-milk protein when determined by the same technique by Henry, Houston, Kon & Osborne (1939) and Henry, Kon, Lea & White (1948). It also lies between the values of 85.5 for yeast and 93.4 for milk found by Mitchell (1923-4b) using his balance-sheet method and feeding the proteins at a level of 5 % in the diet.

The value of 73.2 for the true digestibility is somewhat low but it is strikingly similar to that of 74 obtained by Mitchell (1923-4b) for the crude protein of yeast.

SUMMARY

1. Rumen liquid (130 l.) was incubated at 39° for 4 hr. in 57 batches of 2-3 l., each with 1 % maltose and 0.05 % urea, and the bacterial sludge separated by centrifugation in a Sharples centrifuge. The sludge was dried by successive treatments with ethanol and ether.

2. The dried preparation (461 g., of which 7.5 % was moisture) contained on a dry weight basis 44.4 % protein and 7.1 % ash. Its analysis is compared with corresponding figures for fodder yeast and linseed cake.

3. It was calculated that about 58 % of the bacterial protein was present in the liquid as it was removed from the rumen. The remaining 42 % was synthesized during incubation. The preparation did not appear to be contaminated with feeding-stuff or feeding-stuffs protein to any significant degree.

4. The biological value and the true digestibility of the preparation were determined using twelve young female rats. The values obtained were 88.3 and 73.2 respectively.

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The Utilization of Non-protein Nitrogen in the Bovine Rumen

6. THE EFFECT OF METALS ON THE ACTIVITY OF THE RUMEN BACTERIA

By M. L. McNAUGHT, E. C. OWEN AND J. A. B. SMITH

The Hannah Dairy Research Institute, Kirkhill, Ayr

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In their investigations on the utilization of urea in the bovine rumen Pearson & Smith (1943*b*) and Smith & Baker (1944) concluded that the bacteria which normally inhabit the rumen assimilate significant amounts of non-protein nitrogen (N.P.N.) and convert it to protein. The conclusion was based on the observed diminution of N.P.N. and the formation of protein in the liquid portion of rumen contents during incubation *in vitro* at 39° after addition of carbohydrate. This *in vitro* technique lends itself to the investigation of both the organic and mineral nutrilites which may be required by rumen bacteria, and in the present communication a description is given of experiments carried out to determine the effect of some of the more common metals on the activity of the rumen bacteria. The subject may have wide practical implications, for it is well known that diseases of stock resulting from mineral deficiencies are widespread and that the requirements of ruminants for some metals seem to be larger than those of other animals, a fact which suggests that the additional requirements may be needed for the intense bacterial activity which is characteristic of the normal rumen. The importance to livestock of metals such as copper and cobalt has been widely discussed in the last few years, and reviews on the subject have been published by Green (1944) and Owen (1947). In Western Australia copper deficiency leads to 'enzootic ataxia' in lambs and to 'falling disease' in cattle, and in Britain to 'sway-

back' in sheep. In many parts of the world diseases of cattle and sheep, such as the 'enzootic marasmus' of Western Australia and the 'vinquish' of Scotland are now known to be caused by cobalt deficiency. Not only deficiency but also slight excess of certain metals in the diet of ruminants may lead to disease. Perhaps the best known example of this is the 'teart' of Somerset, to which only ruminants are subject, and which occurs in animals grazed on pastures having a high content of molybdenum (Ferguson, Lewis & Watson, 1943).

The work described in the present paper was done in two sections. The object in the first section was to determine whether and at what levels copper, cobalt, molybdenum and iron were toxic to rumen bacteria, and in the second section to determine whether certain metals were essential for bacterial growth in rumen contents. Since the growth of rumen bacteria in pure culture is beset with many difficulties (Owen, 1947; Elsdon & Phillipson, 1948) it is not possible to study the mineral requirements by growing the bacteria in a synthetic medium purposely made deficient in a given element. Moreover, there are grave doubts as to whether results obtained with pure cultures would help much in elucidating the processes occurring in the normal rumen where a mixed population of bacteria and protozoa is always found (Baker, 1943) and where symbiotic relationships of one species of micro-organisms to another almost certainly obtain (Owen, 1948).