The Utilization of Non-protein Nitrogen in the Bovine Rumen

8. THE NUTRITIVE VALUE OF THE PROTEINS OF PREPARATIONS OF DRIED RUMEN BACTERIA, RUMEN PROTOZOA AND BREWER'S YEAST FOR RATS

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Earlier papers (cf. Pearson & Smith, 1943) in this series described the considerable protein synthesis which occurs when rumen liquid, obtained from a bullock with a permanent fistula, is incubated *in vitro* with non-protein nitrogen and readily available carbohydrate. Smith & Baker (1944) later correlated this protein synthesis with increase in numbers of iodophilic bacteria. Subsequent work showed that the protein of a dried preparation of rumen bacteria, approximately 40 % of which had been synthesized during incubation *in vitro*, had, when tested in rats, the high biological value of 88 (McNaught, Smith, Henry & Kon, 1950). Its true digestibility was, however, only 73 %. In reviewing the subject of nitrogen metabolism in the rumen, McNaught & Smith (1947) pointed out that, although the power of the rumen protozoa to synthesize protein had not been specifically demonstrated (Schmid, 1939; Smith & Baker, 1944), it was possible that they served a useful purpose by assimilating bacterial protein and thus rendering it more available to the host. The observation of Usuelli & Fiorini (1938), that growth of chicks was progressively improved by additions to their diet of bacteria and protozoa from the rumen of sheep, is in accord with the hypothesis that rumen protozoa contribute to the nutrition of the host animal. Johnson, Hamilton, Robinson & Garey (1944) found that the protein of rumen protozoa was better digested by the rat than that of rumen bacteria. They did not attach much importance to this finding because of the doubtful purity of their bacterial preparation. They had so little material that they used too few rats to make a satisfactory estimate of the relative nutritive values of rumen protozoa and bacteria. Since a procedure for isolating protozoa in adequate amounts from rumen contents has now been devised, it seemed desirable to repeat the earlier tests on a rumen-bacterial preparation, simultaneously with tests on the protozoa. A third source of microbial protein, dried brewer's yeast, was tested at the same time for comparison. The present communication describes the preparation of the dried protozoa at the Hannah Institute and the biological tests carried out at the National Institute for Research in Dairying.

EXPERIMENTAL

Preparation of bacteria

This was as described by McNaught *et al.* (1950). During the collection period a cow and a bullock with rumen fistulas were maintained on daily rations consisting of 6 lb. of a concentrate mixture (equal parts of dried grass, oats and beans) and 10 lb. hay. Glucose (1 %) instead of maltose was added to the rumen liquid before incubation. An electrical homogenizer was used to suspend the bacteria in ethanol. In the present preparation 118 l. of rumen liquid yielded 283 g. of dry bacteria, an average yield of 2·4 g./l. This preparation was mixed with 53 g. from the previous preparation which had been stored at 0–4° for 5–6 yr.

Preparation of protozoa

Since it is well known that the number of protozoa in the rumen can to a large extent be controlled by the diet of the animal and in particular by the level of dietary protein (Ferber, 1929; Mangold, 1929; Louw & van der Wath, 1943; Williams & Moir, 1951), it was essential to establish and maintain a dense population by a suitable diet. The diet that had been fed to the fistulated animals during the preparation of the rumen bacteria (McNaught *et al.* 1950) was found to be satisfactory. The method of preparation recommended by Johnson *et al.* (1944) did not prove satisfactory in our hands, as we were unable adequately to separate fibre from the protozoa.

The most efficient separation was achieved by allowing the liquid, obtained by squeezing rumen contents through muslin, to stand in cylinders in a deep water bath, thermostatically controlled at 39°. Within a short time (about 15 min.) most of the dark-coloured fibrous material rose to the surface and formed a compact layer. The protozoa formed a whitish layer at the bottom of the cylinder and could be separated by decantation. Preliminary experiments showed that addition of some soluble carbohydrate to the liquid accelerated the separation and tended to give a larger volume of protozoa. Dilution of the rumen liquid also tended to hasten separation. Oxford (1951) has shown that rumen protozoa very rapidly ferment certain sugars

Table 1. *Yields of protozoa from samples of rumen liquid withdrawn from animals at different times throughout the day*

(For experimental details see text.)

Time	Protozoa isolated without added sugar: Dry matter (mg./l.)	N as % dry matter	Yield of N (mg./l.)	Protozoa isolated in presence of added sugar: Dry matter (mg./l.)	N as % dry matter	Yield of N (mg./l.)
Animal I						
7.30 a.m.*	117	8·7	10·2	193	5·5	10·6
9.30 a.m.†	116	8·9	10·3	471	4·5	21·2
10.30 a.m.	231	8·7	20·1	506	3·7	18·7
12.00 midday	324	6·9	22·3	370	4·2	15·5
2.45 p.m.	163	6·2	10·1	248	4·2	10·4
5.00 p.m.	104	6·5	6·7	317	4·2	13·3
Animal II						
7.30 a.m.*	189	9·8	18·4	615	4·2	25·5
9.30 a.m.†	259	9·5	24·4	799	4·7	37·2
10.30 a.m.	478	7·6	36·5	747	4·2	31·5
12.00 midday	300	6·8	20·5	422	3·8	15·9
2.45 p.m.	420	7·0	29·4	436	4·3	18·5
5.00 p.m.	278	7·2	20·0	450	5·3	23·6

* Hay fed after removal of sample.
† Concentrates (3 lb.) given after removal of sample.

and store a glucosan (Masson & Oxford, 1951; Sugden & Oxford, 1952). It was therefore of interest to determine whether more bulky yields of protozoa obtained by addition of glucose did, in fact, contain more protein than protozoa isolated in the absence of glucose. Furthermore, the ability of rumen liquid to ferment sugar varies considerably throughout the day in relation to time of feeding (Oxford, 1951). Consequently, it was important to know whether yields of protozoa obtained by our technique varied in a similar manner. In a preliminary experiment, samples of rumen contents were withdrawn at the times shown in Table 1. After filtration through muslin, the liquid was divided into two portions. One portion was diluted with one-third of its volume of warm water (approx. 39°) and the other with the same volume of 0·5% (w/v) glucose. The liquids were then poured into cylinders and allowed to stand in the bath. When the protozoa had ceased streaming out of the floating, fibrous layer, the liquid was carefully decanted and the well-defined protozoal layer was transferred to a beaker. The liquid was well mixed and allowed to settle twice more to take out any protozoa trapped in the fibrous material during the first settling. The combined protozoal fractions were suspended in cold tap water and allowed to settle in the beaker. This procedure was repeated until the supernatant layer was quite colourless. The subsequent procedure was as for the bacteria, i.e. the protozoa were twice suspended in ethanol and twice in ether. The total yields of protozoa and of protein from the two animals are shown in Table 1.

It is clear that maximum yields are obtained 2–4 hr. after the 7.30 a.m. feed. The addition of sugar to samples obtained during this period resulted in a considerable increase in the total yield of protozoa but had a negligible effect on that of nitrogen. Analyses showed a substantial storage of polysaccharide in the protozoa which had been allowed to settle in the presence of added glucose and, although an increase in the total protozoal nitrogen was not consistently obtained, glucose was subsequently added to all preparations to accelerate the settling of the protozoa. By this technique up to 24 l. of rumen contents, obtained from the two animals 2–4 hr. after feeding, could be processed each day. In all, 878 l. were treated in this way giving a total yield of 447 g. of protozoa, an average yield of 0·5 g./l.

Table 2. *Percentage composition of dried preparations of rumen bacteria and protozoa*

Component	Rumen bacteria	Rumen protozoa
Moisture	11·2	8·2
Protein (N × 6·25)	41·8	26·5
Polysaccharide*	32·0	62·1
Ash	6·7	2·3
Lipid	2·4	1·4
Crude fibre	2·2	1·7
Total	96·3	102·2

* As starch, corrected for pentose.

Dried brewer's yeast

This was obtained from Arthur Guinness Son and Co. (Dublin) Ltd., Park Royal, London, N.W. 10.

Analyses of the preparations

The preparations were analysed for moisture, nitrogen, polysaccharide, ash, lipid and crude fibre. Moisture was determined by the oven method, nitrogen by the Kjeldahl procedure and polysaccharide by the method recommended by the Association of Official Agricultural Chemists (1945) for starch, in which reducing power is estimated after acid hydrolysis. Pentose was also measured and correction made for it in calculation of the polysaccharides. Lipid material was estimated by saponification followed by extraction, ash by ignition and crude fibre by the method of Whitehouse, Zarow & Shay (1945). The results are recorded in Table 2, where it is shown that they account for 96% of the mass of the bacterial preparation. The corresponding figure for the protozoal preparation was 102%, the extra 2% probably being due to an overestimation of polysaccharide.

Table 3. *Individual nitrogen-metabolism data on twelve rats for 6-day periods showing the biological values and true digestibilities of the proteins* (N × 6·25) *of rumen bacteria, rumen protozoa and dried brewer's yeast*

Rat no.*	Protein tested†	Wt. of rat (g.) Initial	Wt. of rat (g.) Final	Diet intake (g.)	N‡ intake (mg.)	Faecal N (mg.)	Urinary N (mg.)	Biological value	True digestibility
				Initial low egg-nitrogen period					
1	—	54	59	45·71	306·3	65·2	58·3	—	—
2	—	53	54	36·47	244·3	76·5	50·6	—	—
3	—	53	61	43·53	291·7	70·0	54·7	—	—
4	—	55	55	30·16	202·1	76·1	52·9	—	—
5	—	55	62	47·29	316·8	84·2	50·1	—	—
6	—	57	64	44·15	295·8	82·6	62·7	—	—
7	—	63	70	46·48	311·4	88·6	54·6	—	—
8	—	61	61	33·64	225·4	68·4	48·1	—	—
9	—	56	64	48·14	322·5	92·6	64·0	—	—
10	—	54	59	44·62	299·0	69·6	60·3	—	—
11	—	49	55	37·77	253·1	60·7	63·9	—	—
12	—	53	61	47·84	320·5	72·1	59·9	—	—
				1st Experimental period					
1	B	64	70	38·97	483·5	200·1	107·2	87·6	71·5
2	P	62	69	36·47	417·9	129·0	116·6	83·6	87·4
3	Y	68	68	31·33	395·0	121·3	137·4	77·5	83·3
4	B	62	66	30·67	380·6	153·2	95·1	88·2	80·0
5	P	70	84	47·48	544·2	125·8	118·4	89·0	92·2
6	Y	73	75	39·32	495·8	155·9	161·7	78·0	83·0
7	B	78	78	33·33	413·5	167·0	108·1	85·4	74·9
8	P	70	84	47·96	549·5	116·2	110·0	90·2	94·5
9	Y	71	71	29·83	376·1	123·8	128·1	81·5	82·8
10	B	66	71	43·49	539·6	187·3	136·1	83·5	78·6
11	P	63	77	48·98	561·3	147·9	163·6	82·9	88·7
12	Y	71	80	48·81	615·5	186·0	188·0	77·9	82·4
				2nd Experimental period					
2	Y	71	79	45·00	567·4	182·0	221·4	66·0	84·4
3	B	70	74	44·50	552·2	229·3	142·1	81·1	73·9
1	P	76	86	49·50	567·2	163·7	191·5	74·0	80·9
5	Y	90	96	57·00	718·8	201·6	278·4	66·7	85·8
6	B	78	86	48·00	595·5	211·7	153·2	82·9	78·7
4	P	72	88	51·00	584·5	140·3	144·7	87·2	97·8
8	Y	88	94	57·00	718·8	217·4	286·6	63·0	82·1
9	B	71	77	47·50	589·3	285·4	177·4	72·7	68·0
7	P	80	93	51·00	584·5	134·7	192·8	76·6	93·5
11	Y	81	91	56·85	716·8	201·4	292·2	68·5	86·4
12	B	82	88	54·50	676·3	277·8	192·9	77·0	72·4
10	P	79	89	55·50	636·0	147·8	209·5	76·6	91·9
				3rd Experimental period					
3	P	83	93	49·99	572·9	174·9	196·4	76·3	87·4
1	Y	93	100	61·98	781·6	266·4	301·2	65·3	81·3
2	B	87	93	54·49	676·1	313·0	165·6	78·6	70·4
6	P	95	108	59·97	687·3	152·5	211·2	80·0	92·8
4	Y	95	102	61·93	780·9	255·1	318·7	65·6	87·1
5	B	103	115	67·99	843·7	365·0	189·5	82·0	70·6
9	P	85	99	59·98	687·4	192·8	206·7	79·2	90·3
7	Y	101	112	62·98	794·3	240·1	146·1	90·7	84·7
8	B	103	117	66·95	830·7	343·8	222·2	75·4	69·2
12	P	99	115	65·97	756·1	165·8	341·5	65·0	93·3
10	Y	99	107	63·99	806·9	275·2	336·4	60·3	80·4
11	B	98	108	63·48	787·5	333·4	220·9	77·8	73·3
				Final low egg-nitrogen period					
1	—	107	113	61·75	413·7	129·3	84·5	—	—
2	—	99	105	53·67	359·6	111·5	64·0	—	—
3	—	98	102	47·25	316·6	103·8	85·1	—	—
4	—	107	107	44·24	296·4	110·3	87·9	—	—
5	—	123	137	77·47	519·0	131·0	94·4	—	—
6	—	116	130	73·48	492·3	123·4	92·6	—	—
7	—	122	136	75·98	509·1	143·4	99·4	—	—
8	—	123	137	75·47	505·6	81·6	93·1	—	—
9	—	108	120	71·33	477·9	152·9	88·1	—	—
10	—	117	131	72·65	486·8	137·8	82·8	—	—
11	—	115	129	73·45	492·1	151·2	96·5	—	—
12	—	124	138	77·46	519·0	140·9	109·0	—	—

* Rat nos. 1, 2, 3 = litter 1; 4, 5, 6 = litter 2; 7, 8, 9 = litter 3; 10, 11, 12 = litter 4.

† B = rumen bacteria; P = rumen protozoa; Y = dried brewer's yeast.

‡ Nitrogen content of diets: low egg-nitrogen, 0·67%; rumen bacteria, 1·24%; rumen protozoa, 1·15%; dried brewer's yeast, 1·26%.

Table 4. *Mean results for groups of twelve rats for the biological values, true digestibilities and net utilization values of the proteins* (N × 6·25) *of rumen bacteria, rumen protozoa and dried brewer's yeast*

Protein	Biological value	True digestibility	Net utilization (biological value × true digestibility/100)
Dried rumen bacteria	81	74	60
Dried rumen protozoa	80	91	73
Dried brewer's yeast	72	84	60
Pooled S.E.	±1·53	±0·95	—

Purity of the preparations

The fibre content of the earlier bacterial preparation, 0·37 %, was taken as an indication of contamination with vegetable material (McNaught *et al.* 1950). The higher value of 2·2 % in the present sample might indicate greater contamination. It should be pointed out, however, that the present value was obtained by the method of Whitehouse *et al.* (1945), which is simpler than the double-digestion method of the Association of Official Agricultural Chemists (1945) used previously. A sample of the first preparation when analysed by the method of Whitehouse *et al.* (1945) gave a value of 2·06 % as compared with 0·37 % by the other method. This rather suggests that it is not true fibre that is being estimated, for the two methods agree well when used to determine fibre in feeding stuffs (Owen, 1946). When examined microscopically, both preparations appeared to contain only traces of fibre.

Microscopic examination of the protozoal preparation showed that it contained a considerable amount of extracellular material. At first this was thought to be contaminating bacteria, but further investigation showed that the material was probably the 'glucosan' granules from ruptured holotrich ciliates reported by Oxford (1951).

Biological tests

The biological value and true digestibility of the proteins of the rumen preparations and of the dried yeast were determined in litter-mate comparisons on four litters of three weanling female rats, at an 8 % level of protein intake, by the method of Mitchell (Mitchell, 1924*a*; Mitchell & Carman, 1926) as used in this laboratory (Henry, Kon & Watson, 1937; Henry, Kon, Lea & White, 1948). In each litter the rats received each diet in turn according to a Latin-square design.

The basal, nitrogen-free diet contained: cane sugar 12, margarine fat 10, potato starch 10, rice starch 64 and salt mixture (de Loureiro, 1931) 4 %. (This salt mixture is the same as that described by Campbell, Olley & Blewett (1949) except that it contains no $ZnSO_4$.) To bring the protein content of this diet to 8 % the following percentages of the test substances were added to it at the expense of rice starch: rumen bacteria, 19·0; rumen protozoa, 31·2; brewer's yeast, 20·6.

The results of the test are shown in Tables 3 and 4.

DISCUSSION

The estimates of the biological value and true digestibility of the bacterial protein agree satisfactorily with those previously obtained by us for a different sample of rumen bacteria (McNaught *et al.* 1950). The biological value of 81 now obtained is not essentially different from the earlier one of 88; we have noted similar variations in tests done at different times on the same sample of dried skim milk (cf. Henry, Kosterlitz & Quenouille, 1953). The present value is closely similar to those of 78 and 79 reported for two samples of bacteria isolated from the rumen of sheep by Reed, Moir & Underwood (1949) in Western Australia. The true digestibilities of 74 and 73, respectively, found now and in our previous test, are higher than those reported by the Australian workers (65 and 62). Although the biological value of the protozoal protein (80) does not differ from that of the bacteria, the true digestibility of 90 is markedly higher. The biological value of 72 found for the yeast is lower than the values for the bacteria and protozoa but falls within the range of 45–86 reported for yeasts in the literature (Goyco & Asenjo, 1947, 1949; Hughes & Hauge, 1945; Mitchell, 1924*b*; Sure & House, 1949). The true digestibility of 84 for the yeast is intermediate between the figures for bacteria and protozoa; it falls well within the range of 80–90 reported for yeasts by the above workers. It is of interest that the net utilization (biological value × true digestibility/100) for both the bacteria and the yeast is 60 %. The protozoa, with their higher digestibility, have a value of 73 %. Thus, in these tests, protozoa were superior to both rumen bacteria and yeast.

For many years the role of the protozoa in ruminant metabolism has been under investigation, and one of us (Owen, 1947*a*, *b*, 1951, 1954), in reviewing the status of rumen micro-organisms, has summarized the more recent work on the assessment of the value of protozoa. Becker (1929) and Becker & Everett (1930) observed that sheep defaunated with copper sulphate thrived better than untreated controls, suggesting that the protozoa were of no value to the host. Copper sulphate, however, not only would remove the protozoa in the rumen but would also increase the animal's thrift by acting as a vermifuge. There is, in addition, the possibility that Becker's control animals were not receiving optimum amounts of copper, the essential nature of which element was not then fully appreciated. Van der Wath & Myburgh (1941), using the

same defaunation technique, could not demonstrate an essential role for the protozoa in cellulose digestion, although it is known from the work of Hungate (1942, 1943) that at least one rumen protozoan (*Diplodinium* sp.) can digest cellulose. More recently, Sugden & Oxford (1952) found that the holotrich ciliates of the sheep's rumen could not use soluble starch for growth, though they could use inulin or a bacterial levan. It is therefore doubtful to what extent the protozoa play a part in the carbohydrate metabolism of the rumen. Their part in nitrogen metabolism also is rather obscure. The present investigation has indeed shown that the protozoa provide the host with a readily digestible protein of high biological value. Nevertheless, the final assessment of the benefit, if any, of its alimentary protozoa to the host can be made only after it is known from what nitrogen source the protozoa elaborate protein, and at what rate this protein becomes available through autolysis and digestion. Whether rumen protozoa can use non-protein nitrogen for protein synthesis is a matter for further study. They may depend entirely on the protein of the host's fodder or of the rumen bacteria, or their growth requirements may be satisfied by a less complex nitrogen source, such as amino acids, or even inorganic nitrogen in the presence of an organic carbon source (Hall, 1941). Whereas the biological values of the bacteria and protozoa have been shown to be the same, lower values of 60–70 have been reported for the usual feeding stuffs such as grass, oats, etc. (Block & Mitchell, 1946; Bartlett *et al.* 1938). Utilization of such food protein would involve some amino acid synthesis to yield protein of the higher biological value found in the present experiments. Thus the alimentary protozoa of the ruminant may well enhance the value of food or of bacterial protein by increasing its digestibility.

Though the yield of protozoa in the present experiments (0·5 g./l. rumen contents) appears to be small, the method of isolation was by no means quantitative nor did it separate the smaller protozoa. Estimates of the amount of protozoal material in the rumen have been discussed by Mangold (1929), who quotes Ferber (1929) as having found 4·4–8·7 % of protozoa in the dry matter of rumen contents. He also quotes Schwarz (1925) as estimating that 20–30 % of the nitrogen in the rumen contents of slaughterhouse cattle was due to protozoa. This estimate was made by assuming that all the nitrogen brought into solution by pouring a pepsin-hydrochloric acid mixture through a filter paper containing rumen contents was protozoal. Masson & Oxford (1951) could find only 1 % of protozoal starch ('glucosan') in a sheep's whole rumen contents but this estimate, like those of Ferber (1929) and of Schwarz (1925), gives no measure of the rate at which the total protozoal products are passing on to the abomasum or are being autolysed in the rumen itself. If Schwarz's results are correct or even if the lesser ones of Ferber are a better estimate, then the difference of utilization between protozoal and bacterial protein may well be of importance to the host.

SUMMARY

1. A sample of dried bacteria (283 g.) was prepared from bovine rumen liquid, by the method of McNaught *et al.* (1950). A sample of dried protozoa (447 g.) was prepared from rumen contents by straining them through muslin, diluting the strained liquid with glucose solution and allowing this liquid to settle at 39°. The protozoal layer was separated by decantation. These preparations, when dried, contained 41·8 % and 26·5 % of crude protein, respectively.

2. The biological value and true digestibility of the bacterial and protozoal protein were determined at an 8 % level of protein intake on groups of twelve young female rats by the balance-sheet method. Dried brewer's yeast was also included in the trials. Biological values of 81, 80 and 72 were obtained for the proteins of bacteria, protozoa and yeast, respectively. The corresponding values for true digestibility were 74, 91 and 84 %.

3. These results suggest that the conversion of bacterial or dietary protein into protozoal protein in the rumen is advantageous to the host animal.

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Studies on the Biosynthesis of Blood Pigments

1. HAEM SYNTHESIS IN HAEMOLYSED ERYTHROCYTES OF CHICKEN BLOOD

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In recent years, the isotope tracer method has been used extensively to study the biosynthesis of haem. This has shown that glycine nitrogen probably supplies all four haem nitrogens (Wittenberg & Shemin, 1949; Muir & Neuberger, 1949), and that glycine-α-carbon is used for the four methene bridges and for one carbon atom in each pyrrole nucleus (Muir & Neuberger, 1950; Wittenberg & Shemin, 1950). The remainder of the carbon atoms can be furnished by acetate (Shemin & Wittenberg, 1951). For these studies either whole animals or intact avian erythrocytes were used. For detailed studies of the mechanism, a system without cell-permeability barriers would be of obvious advantage. The synthesis can be studied in rabbit bone marrow 'homogenates' (Altman, Salomon & Noonan, 1949) and in rabbit-spleen 'homogenates' (Altman & Salomon, 1950). It appeared to us that, if active preparations could be obtained, haemolysed avian erythrocytes should provide a particularly convenient system.

In the present work, conditions are described for the preparation, from the blood of domestic fowls, of completely haemolysed erythrocytes which retain a high proportion of the synthetic activity of the intact cells, as measured by the use of [α-^{14}C]-glycine. The rate of haem synthesis has been studied under various conditions, including the presence of some haemopoietic factors. It has also been possible to show a net increase of free porphyrin on incubation. A preliminary report of this work has been made (Falk & Dresel, 1952). Haem synthesis has been obtained in similar preparations by London & Yamasaki (1952).

MATERIALS

The blood used was taken from normal adult domestic fowls (*Gallus domesticus*) pooled for marketing. Approximately 500 ml. blood was collected into 2 ml. 0·9 % NaCl, containing 15 mg. penicillin G, 15 mg. streptomycin and 20 mg. heparin, and was used the same day. In one experiment, blood from vitamin B_{12}-deficient, 4-week-old chicks, and from the corresponding controls, was used. The parent hens had been on a diet containing all-vegetable protein for about 18 months. The 'B_{12}-deficient' chicks were reared on a B_{12}-free diet (Coates, Harrison & Kon, 1949), whereas the