RUMINAL FLORA STUDIES IN THE SHEEP

IV. THE INFLUENCE OF VARYING DIETARY LEVELS OF PROTEIN AND STARCH UPON DIGESTIBILITY, NITROGEN RETENTION, AND THE FREE MICROORGANISMS OF THE RUMEN

By V. J. WILLIAMS,* M. C. NOTTLE,* R. J. MOIR,* and E. J. UNDERWOOD*

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Summary

Data are presented from a replicated feeding trial with sheep, designed to determine the influence of varying dietary intakes of starch and protein upon digestibility, protein utilization, and the numbers and types of "free" bacteria in the rumen.

The main trial contained nine diets composed of oaten chaff to which wheaten starch and wheat gluten were added so as to provide three levels of starch (nil, medium, and high) and three levels of protein (low, medium, and high).

The concentration of free microorganisms in the rumen was found to be markedly affected both by the levels of dietary protein and by the levels of dietary starch, but the extent to which either of these constituents affected this concentration was greatly influenced by the proportion of the other constituent present in the diet.

In low-protein diets, increases in the intake of starch were found to result in a highly significant reduction in the rumen counts as well as a marked change in the types of organisms present. Where the protein contents of the diets were higher no such effects were evident.

In diets containing medium or high levels of starch, increases in the intake of protein were found to result in highly significant increases in the rumen counts. No such effects were evident in starch-free diets.

At all levels of starch the addition of protein was found to increase significantly the digestibility of the dry matter of the diets and the true digestibility of the protein as well as the rumen counts, but the addition of starch to low-protein diets markedly reduced these counts without influencing significantly the digestibility of the dry matter.

These findings are compared with the results of Moir and Williams (1950) and others, and the conclusion reached that the proportion of *total*, as distinct from *added*, dietary protein converted to bacterial protein in the rumen is not relatively constant, as earlier claimed, but diminishes sharply as the intake of protein increases.

I. INTRODUCTION

In an earlier experiment by the authors (Moir and Williams 1950) an extremely high correlation ($r = +0.98$) was found between the levels of protein intake and the concentrations of free microorganisms in the rumen. The basal ration in this experiment consisted of oaten hay and wheaten starch and contained only 3.7 per cent. protein ($N \times 6.25$). The protein content of the

* Institute of Agriculture, University of Western Australia, Nedlands, W.A.

basal ration was increased by substituting increasing proportions of the starch with equal weights of casein until a ration containing 18 per cent. protein was obtained. In this way the protein content of the five rations used varied from 3.7 to 18 per cent. but their dry matter, crude fibre, and gross energy contents remained unchanged. It is clear, however, that the higher the protein content of rations the lower were their starch contents. It may therefore be asked whether the concentrations of ruminal organisms obtained were a reflection of the higher protein levels of the substituted basal rations or whether they were also influenced by the lower starch levels of these rations. In this paper are presented the results of experiments in which both starch and protein were varied independently.

Considerable overseas evidence exists concerning the influence of supplements of protein and of starch and other carbohydrates upon the digestibility of roughages by ruminants but the effects produced have seldom been related to changes in the ruminal population. Gallup and Briggs (1948) increased the dry matter digestion of prairie hay with cottonseed meal, although Watson *et al.* (1947) found no such effect from additions of soybean oil meal to timothy hay. Burroughs *et al.* (1949*a*) showed that various high-protein feeds restored the digestibility of poor-quality roughages, in the presence of a high proportion of starch. Protein supplements without added starch produced no such effects. In a later study these workers (Burroughs *et al.* 1950) confirmed their earlier findings and extended them to include some bacteriological studies. They found a close correlation between the numbers of ruminal bacteria and the degree of roughage digestion. Where the roughage (ground corncobs and chopped alfalfa hay 4:1) was fed alone digestion was relatively high and the ruminal population high. Addition of starch alone resulted in a marked depression of both digestibility and ruminal population. Addition of starch and casein together restored the digestibility of the roughage and the numbers of ruminal bacteria to levels similar to those of the unsupplemented roughage. It is unfortunate that these workers did not include a diet in which the roughage was supplemented with casein only.

Hamilton (1942) showed that the addition of glucose reduced the digestibility of protein and of fibre in rations for sheep. Burroughs *et al.* (1949*b*) obtained a substantial decrease in roughage digestion by steers by the addition of starch to poor-quality roughages but not with good-quality alfalfa hay. They claimed that the higher protein content of the alfalfa was not the factor causing the difference in the starch effects and suggested that "the variable influence of starch upon roughage digestion can best be explained on the basis of adequate or inadequate nutrients (other than protein) for the growth of microorganisms in the alimentary tract." Louw and van der Wath (1943) added meatmeal to a ration of poor prairie hay and obtained a highly significant increase in the numbers of ruminal bacteria. Replacement of varying proportions of meatmeal with maize resulted in minor increases in the counts until the maize supplements reached 150 g. This increased bacterial population did not improve cellulose digestion. Further addition of maize in the absence of meatmeal caused a decline in bacterial numbers and a significant decline in cellulose digestibility notwithstanding the fact that the N intake increased in these latter diets.

II. Experimental

(*a*) *Design of Experiment*

The feeding trial was designed as a balanced lattice, as set out in Table 1, containing four blocks of replicates of each of nine dietary treatments. The treatments were triplicated at random within each block and each triplicate was allotted at random to one of 12 sheep.

Table 1

EXPERIMENTAL DESIGN — ORDER OF FEEDING DIETS

Sheep No.	Diet No.		
339	2	0	1
338	5	3	4
344	6	8	7
337	3	6	0
332	7	4	1
345	5	8	2
340	8	4	0
341	6	5	1
349	7	3	2
342	5	7	0
347	3	8	1
343	4	2	6

(*b*) *Experimental Animals*

Twelve Merino wethers were selected for evenness of age and appearance from a larger group of similar breeding. Each of the experimental animals weighed about 90 lb. and was between 2 and 2½ years of age.

(*c*) *Diets*

The composition of the nine diets used was as set out in Table 2. They were designed to provide three levels of starch and three levels of protein, with oaten chaff as the roughage in all cases.

It was found impossible to restrict the diets to the three main constituents, oaten chaff, wheaten starch, and wheat gluten because of serious difficulties in persuading the sheep to consume the prescribed amounts of certain types of rations and of preventing them from selecting particular parts of others. Accordingly a proportion of wheaten grain (ground) and wheaten bran was included in some of the diets and in some also a small amount of molasses was added as a binding agent. In all diets, except 0, 1, and 2, the concentrates were sprayed with water while being mixed in a concrete mixer and then dried in an oven at 55°C. in a current of air. In diets 1 and 2 the starch was drenched daily as a suspension in its own weight of water because the sheep refused to consume it voluntarily.

It is clear from Table 2 that the diets can be compared in several ways. Thus a comparison of the data from diets 0, 3, and 6 enables an assessment to be made of the influence of three different levels of protein in starch-free or virtually starch-free diets; a comparison of diets 1, 4, and 7 of similar levels of protein at medium starch intakes; and of 2, 5, and 8 of these levels of protein at relatively high starch intakes. Alternatively a comparison of the data from diets 0, 1, and 2 enables an assessment to be made of the influence of three levels of starch in low (3.5-5.2 per cent.) protein diets; of 3, 4, and 5 of similar levels of starch in medium (8.2-10.0 per cent.) protein diets, and of 6, 7, and 8 of these levels of starch in diets of higher (10.6-13.1 per cent.) protein content.

TABLE 2

COMPOSITION AND INTAKE OF DIETS — G. DRY MATTER PER DAY

Diet	Oaten Chaff	Wheat	Wheaten Starch	Wheaten Gluten	Wheaten Bran	Molasses	Ground Limestone	Rock Salt	Dry Matter Intake (g./day)	Percentage N	Percentage Crude Protein	Intake Protein (g./day)	Intake Starch (g./day)
0	270								270	0·83	5·2	14·0	0
1	270		99						369	0·63	3·9	14·2	99
2	270		149						419	0·56	3·5	14·6	149
3	495			41	58		3	3	600	1·60	10·0	60·0	0*
4	495	74	49	41		18	3	3	683	1·48	9·3	63·4	99
5	495	74	99	41		18	3	3	733	1·31	8·2	60·1	149
6	495			80	58		3	3	639	2·10	13·1	83·7	0*
7	495	74	49	80		18	3	3	722	1·83	11·4	82·3	99
8	495	74	99	80		18	3	3	772	1·70	10·6	81·8	149

* Excluding small amount present in wheaten bran.

(*d*) *Treatment of Animals and Samples*

Each feeding period extended over 24 days, the last 10 days of which constituted the collection period. Between treatments all sheep were given a "stabilizing" period of 24 days on diet 4 (medium protein, medium starch) to ensure a comparable nitrogen status and to minimize any effects of previous treatments on the ruminal population. The sheep were kept in metabolism crates for the treatment periods and in separate pens for the stabilizing periods.

The whole of the prescribed daily ration was fed each day at 9 a.m., except that in rations 1 and 2 the starch was drenched at 10 a.m., i.e. 1 hr. after the chaff was fed. Adequate tap water was before the sheep at all times and the sheep were drenched once a week with a vitamin A and D concentrate. This was carried out at least 7 days before the rumen contents were sampled.

Urine, faeces, and any feed residues were collected daily immediately before feeding and treated as described by Moir and Williams (1950). On the seventh

and ninth days of each collection period samples of rumen contents were withdrawn by stomach tube and treated as described by Moir (1951).

The sheep were weighed on the first, thirteenth, and twenty-fourth days of each treatment. The mean of the last two weights was used in the calculation of the biological values of the protein of the diets.

III. Results

The mean results of the digestibility determinations, nitrogen metabolism data, and ruminal microorganism counts are presented in Table 3. The statistical significance of the results of the various treatments was determined and

Table 3

MEAN DIGESTIBILITY, NITROGEN METABOLISM, AND RUMINAL FLORA DATA AT DIFFERENT INTAKES OF PROTEIN AND STARCH
(Each value the mean of 4 sheep)

	Low Protein					
	N Intake (g./day)	True Digest. N (%)	Calc. Biol. Value N	Urinary N/ Absorbed N (%)	Digest. Dry Matter (%)	Rumen Bacteria (millions per cu. mm.)
No starch				Diet 0		
	2·2	83	61	96	52	49
Medium starch				Diet 1		
	2·3	85	92	68	58	27
High starch				Diet 2		
	2·2	81	92	78	55	21

	Medium Protein					
	N Intake (g./day)	True Digest. N (%)	Calc. Biol. Value N	Urinary N/ Absorbed N (%)	Digest. Dry Matter (%)	Rumen Bacteria (millions per (cu. mm.)
No starch				Diet 3		
	9·6	92	72	42	63	57
Medium starch				Diet 4		
	9·7	97	75	38	67	47
High starch				Diet 5		
	9·5	93	84	33	67	44

TABLE 3 (*Continued*)

	High Protein					
	N Intake (g./day)	True Digest. N (%)	Calc. Biol. Value N	Urinary N/ Absorbed N (%)	Digest. Dry Matter (%)	Rumen Bacteria (millions per cu. mm.)
No starch				Diet 6		
	13·5	96	65	45	69	59
Medium starch				Diet 7		
	13·3	97	70	37	73	58
High starch				Diet 8		
	13·0	94	64	46	72	54

* The individual data from which these means were calculated may be obtained on request from the Director, Institute of Agriculture, University of Western Australia, Nedlands, W.A.

is discussed below. For the concentration of ruminal microorganisms and the true digestibility of the nitrogen of the diets the results were analysed as designed, i.e. as a balanced lattice, but the lattice was not effective for the calculated biological values of the dietary nitrogen and the dry matter digestibilities. These were analysed as a randomized block. In these determinations, the variability between sheep was as small as, or smaller than, the error term. Tests for significance were either by the variance ratio or Student's *t* test.

(*a*) *Concentration of Free Ruminal Microorganisms*

Effect of starch.—In the low-protein diets there is a marked effect of starch. The mean concentration of 49,000,000/cu. mm. in diet 0 is significantly higher than that of diet 1 (27,000,000/cu. mm.) at the 5 per cent. level and of diet 2 (21,000,000/cu. mm.) at the 1 per cent. level. In the medium-protein diets there is apparently a similar, though much less marked effect since the "mean counts" fall from 57,000,000 to 47,000,000 and then to 44,000,000/cu. mm. in diets 3, 4, and 5 respectively. These differences, however, are not significant. In the high-protein diets the mean counts are very similar in the three diets (6, 7, and 8) and there is no significant effect of the three levels of starch.

Effect of protein.—Where the rations contain no starch (diets 0, 3, and 6) increasing the protein intake has had no significant effect in increasing the density of the ruminal population. The mean counts of these rations show a small increase but the differences are not significant. Where starch is included, however, either at the medium or high level, increasing the protein has resulted in significant increases in the bacterial counts. Thus the mean counts for diets 4 and 5 (47,000,000 and 44,000,000/cu. mm.) are significantly higher than those

of diets 1 and 2 (27,000,000 and 21,000,000/cu. mm.) at the 5 per cent. level, and the means of diets 7 and 8 (58,000,000 and 54,000,000/cu. mm.) are significantly higher than those of diets 1 and 2 at the 1 per cent. level. The differences between the mean counts of diets 7 and 8, although higher than those of diets 4 and 5, are not significantly different, indicating that raising the proportion of protein from 9.3 to 11.5 per cent. or from 8.2 to 10.6 per cent. (see Table 2) has not significantly increased the concentrations of the ruminal microorganisms.

(*b*) *Microscopic Characterization of the Microorganisms*

Microscopic characterization of the organisms present in the rumen samples was based on the descriptions by Moir and Masson (1952). A total of 21 group types were identified on the basis of their morphological characteristics in Gram-stained smears. Animals maintained on diet 0 generally showed a high proportion (over 85 per cent.) of Gram-negative organisms, with fewer Gram-positive forms than samples from the sheep fed on the diets containing added protein or starch.

The most notable features of diets 1 and 2 were the increased proportion of Gram-positive organisms and the presence of a pleomorphic Gram-variable rod in long chains clustered about the starch granules. The same organism appeared to a very limited extent in a few animals on diets 7 and 8.

Burroughs *et al.* (1950) have described a similar condition under comparable dietary treatment. Both in their work and in the present experiment, the total bacterial count was very significantly reduced in these cases. Further bacteriological and biochemical work under similar conditions is necessary to assess the biological significance of this relationship.

All other diets produced a high proportion of Gram-positive organisms, more particularly when the total count was low. It is noteworthy that in three of the 12 sheep used, the large *Selenomonas* species, including the organism described by Quin (1943), persisted throughout the experiment on all three dietary treatments and through two treatments in a further two animals. This is difficult to explain, as in preliminary work, when the sheep were maintained on diet 4 in an attempt to standardize the ruminal population, these organisms were eliminated in the rest of the animals within 2 weeks.

(*c*) *Dry Matter Digestibility*

Inspection of Table 3 reveals that the addition of starch, at each level of protein, has brought about little change in the mean dry matter digestibility of the diets. The small differences between diet 0 and diets 1 and 2, between diet 3 and diets 4 and 5, and between diet 6 and diets 7 and 8 are not significant. On the other hand, at all levels of starch feeding the addition of protein has significantly increased the mean dry matter digestibility. Thus the dry matter digestibility of diet 3 (63 per cent.) is significantly higher than that of diet 0 (52 per cent.), of diet 4 (67 per cent.) than that of diet 1 (58 per cent.), and of diet 5 (67 per cent.) than that of diet 2 (55 per cent.), all at the 5 per cent. level. The mean dry matter digestibilities of diets 6, 7, and 8

are significantly higher than those of diets 0, 1, and 2 respectively at the 1 per cent. level. The overall means of dry matter digestibility of the low-, medium-, and high-protein diets are 55, 61, and 71 per cent. These overall means are significantly different.

(*d*) *Nitrogen Balance Data*

In the calculations of the biological values and true digestibility of the nitrogen of the rations a figure of 0.44 g. N per 100 g. food consumed was used for the metabolic faecal N. This is very close to the average figure of 0.5 g. as found by Blaxter and Mitchell (1948), which was found to be somewhat too high for many of our sheep. Endogenous urinary N was assumed to be 0.033 g. N per kg. body weight, as found by Harris and Mitchell (1941).

True digestibility of N.—Inspection of the mean values for the true digestibility of N of the various diets (Table 3) reveals that the proportion of starch in the ration has not influenced these values at any one of the three levels of protein. There is, however, a significant effect of protein at each level of starch. The mean true digestibility of the N of the high-protein diets (6, 7, and 8) is significantly higher, at the 1 per cent. level, than that of the corresponding low-protein diets (0, 1, and 2). The values for the medium-protein diets (3, 4, and 5) are also significantly higher than the corresponding low-protein diets at the 1 per cent. level. The mean true digestibility of the N of the three low-protein diets is 83 and that of the six diets with added protein is 95.

Urinary N as percentage of absorbed N.—The feeding of starch has had no significant effect on these values at any level of protein. Increasing the protein content of the rations from low to medium or from low to high has, however, again had a significant effect at the 1 per cent. level. The percentage of the absorbed N appearing in the urine decreased from an overall mean of 81 in the three low-protein diets to a mean of 40 in the six diets containing added protein. Raising the protein content from medium to high has not significantly altered the position.

Biological values.—The mean figures for the calculated biological values of the N of the various diets (Table 3) indicate an effect of both starch and protein, although the effect of starch is confined to the low-protein diets (0, 1, and 2). The mean of 92 for the medium- and high-starch levels of diets 1 and 2 is significantly higher ($P < 0.01$) than the value of 61 for diet 0. It is obvious, however, that there can be no true assessment of the biological value of the protein of diet 0 because the low protein and available energy of this diet would result in considerable tissue catabolism and because the high rumen population under these conditions has probably resulted also in amino acid deamination with consequent ammonia N loss from the rumen.

No effect of the starch is apparent in the medium- and high-protein diets. The mean biological values of the low- and medium-protein diets are not significantly different but the means of both these diets are highly significantly greater ($P < 0.01$) than that of the high-protein diets. This relatively high level of protein feeding (10.6-13.1 per cent. on the dry basis) has obviously exceeded the maximum required for the full utilization of the absorbed N.

IV. Discussion

It is apparent, from the experiments described, that the concentration of free microorganisms in the rumen is affected by the levels of both protein and starch in the ration. It is apparent, further, that the extent to which either constituent affects this concentration is greatly influenced by the proportion of the other constituent present. Thus, in low-protein diets, increases in the intake of starch result in a marked reduction in the rumen counts accompanied by marked changes in the types of organisms present. Where the diets contain a higher proportion of protein no significant quantitative or qualitative changes in the rumen population occur as the result of increased intakes of starch. Similarly, the effect of protein is influenced by the starch intake. Increases in the level of protein do not significantly alter the rumen counts where the rations are starch-free but increases in protein intake markedly increase these counts where the rations contain medium or high amounts of starch.

It is of interest to compare these findings with those of the earlier experiment of Moir and Williams (1950) in which similar rations were used, except that the protein supplement was casein rather than wheat gluten. It is obvious from Table 4 that, where starch is included in the diets, the values obtained in the two experiments for the concentration of free microorganisms in the rumen are very similar.

Table 4

INFLUENCE OF PROTEIN INTAKE ON THE CONCENTRATION OF RUMINAL BACTERIA

Moir and Williams (1950)				Present Experiments			
Group No.	Starch Intake (g./day)	Protein Intake (g./day)	Rumen Counts (millions/ cu. mm.)	Group No.	Starch Intake (g./day)	Protein Intake (g./day)	Rumen Counts (millions/ cu. mm.)
1	173	15·0	29	1	99	14·2	27
4	99	62·5	55	4	99	63·4	47
5	73	76·9	61	7	99	82·3	58

In view of the relatively constant increase in the numbers of microorganisms with increasing protein intakes that was obtained in the earlier experiment, it was suggested that a constant proportion of the food protein was converted to bacterial protein. This is incorrect on the basis of the *total* protein ingested although, if the concentration of ruminal bacteria is a measure of the proportion of dietary protein converted to bacterial protein, the proportion of *added* protein converted is relatively constant. It is obvious, both in the present and in the earlier experiments, that on this basis the proportion of total dietary protein converted to bacterial protein diminishes sharply as the intake increases. On the same basis the addition of starch to diet 0 has approximately halved this conversion.

The high rumen populations obtained on diets consisting solely of relatively poor roughage are of considerable interest and confirm the findings of Burroughs *et al.* (1950) in this respect. These workers found a high positive correlation between the numbers of ruminal bacteria and the degree of roughage digestion. This is similar to our own data on dry matter digestibility since the addition of protein to starch-containing diets has significantly increased both the mean dry matter digestibility and the mean bacterial density of the rumen. No such correlation exists, however, in the data from diets 0, 1, and 2. The addition of starch has markedly reduced the rumen population without influencing significantly the digestibility of the dry matter. No explanation of these differential effects is yet available although changes in the fixed ruminal flora may be of possible significance. A further problem of great importance raised by these findings is the minimum N requirements of the rumen for bacterial proliferation and function. The high rumen population obtained on the diets consisting only of poor-quality roughage indicates that these requirements are extremely low or at least lower than the minimum requirements of the sheep as a whole for the most effective use of the dry matter and the protein of the diet.

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