RUMINAL FLORA STUDIES IN THE SHEEP

II. THE EFFECT OF THE LEVEL OF NITROGEN INTAKE UPON THE TOTAL NUMBER OF FREE MICROORGANISMS IN THE RUMEN

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Summary

A method of obtaining ruminal samples for the investigation of the "free" microorganisms of the sheep's rumen by means of a stomach-tube is described.

The total numbers of microorganisms per unit volume obtained in this way were found to be lower than in samples taken directly from whole-rumen contents. The correlation between the counts for the two series of samples and the constancy of the difference is considered to justify the stomach-tube method as a means of assessing changes in the number of microorganisms in the rumen.

The data are presented from a feeding trial designed to determine the effects of varying intakes of protein upon the number of ruminal microorganisms. In this trial five sheep were fed five levels of protein derived from casein and oaten hay. The levels of protein consumed were approximately 3, 6, 9, 10, and 12 per cent. of the dry matter of the ration.

An extremely high correlation ($r = +0.98$) was found between the levels of intake of protein and the numbers of microorganisms in the rumen. It was concluded that, under the conditions of the experiment, the number of organisms was determined by the protein intake.

The regressions for the numbers of organisms against protein nitrogen are presented. These indicate that a relatively constant proportion of the ingested protein (of the order of 50 per cent.) was converted to bacterial protein.

These findings, together with the "true" digestibility and biological value of the protein of each ration, are discussed in relation to protein metabolism in the ruminant.

I. Introduction

During the last decade the functional importance of the indigenous microflora and microfauna of the rumen has been fully recognized and considerable attention directed to elucidating their precise roles in ruminant metabolism. The spectacular success achieved by these animals in utilizing insoluble polysaccharides as a source of energy has naturally attracted many investigators, and notable advances have been made in our understanding of the parts played by the ruminal microorganisms in this process. The position with respect to the utilization of the nitrogenous constituents of the feed is much less satisfactory, although it is obvious that the whole microbial population must participate in some way in protein metabolism since ingested protein is normally its major source of nitrogen.

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The fact that ruminal microorganisms must obtain all or most of their nitrogen from the food suggests that, in the presence of adequate available energy and other nutrients, the intake of nitrogen will determine the extent of the proliferation of these organisms. Little direct experimental evidence upon this point was available before the experiments described in this paper. Smith and Baker (1944) showed that, where ammonium salts or urea were fed as the main source of nitrogen, multiplication was limited by energy supplies. van der Wath (1942) found that the addition of dextrinized starch to a basal ration of poor wheat straw did not raise the ruminal bacterial count, whereas the further addition of urea raised it by 74 per cent. Replacement of the urea by fishmeal increased the count to three times the original figure. van der Wath and Myburgh (1941) demonstrated a considerable seasonal fluctuation in the numbers of ruminal bacteria in grazing sheep in South Africa. They suggested that the protein content of the pasture influenced the numbers considerably. This suggestion was later supported by the work of Louw and van der Wath (1943), who added meatmeal to a ration of poor prairie hay containing 3 per cent. protein 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 supplement reached 150 g. Further additions of maize in the absence of meatmeal, and particularly when 300 g. were fed, caused a decline in bacterial numbers.

These findings indicate that the level of protein ingested can affect the numbers of microorganisms within the rumen. The results of an experiment designed to reveal whether any direct quantitative relationship exists between protein intake and numbers of ruminal microorganisms in the sheep are presented in this paper.

II. Ruminal Sampling Technique

(*a*) *The Sampling Method*

The use of a stomach-tube as a means of obtaining ruminal samples, rather than fistulated animals, was investigated. The path taken by tubes passed into the oesophagus was described by Watson and Jarrett (1945), who showed that the tube, unless accompanied by a liquid, passes into the rumen or reticulum.

Tubes of 6 to 7 mm. diameter were introduced through the nostril and were found to pass readily into the oesophagus. Much larger tubing can be passed by way of the mouth. The minimum diameter that could be used without blocking, particularly in grazing animals, was 6 to 7 mm. After carefully introducing the tube 27 to 30 in., a suction pump was attached and negative pressure applied. A trap in the line was employed so that the ruminal sample was withdrawn directly into a large sample bottle. A quantity of this material was immediately diluted with half its volume of 50 per cent. formalin. In this state, samples remained unchanged for a considerable period.

While it was recognized that this method of sampling is not ideal, it was considered that the samples taken should reflect any changes taking place in the total numbers of ruminal microorganisms. Baker (1943) showed that, in the ox, the proportion of solid to liquid fraction diminishes progressively with the depth. Further, the efficiency of the stomach-tube in gathering representative samples was strongly doubted by Gall, Stark, and Loosli (1947), who found that the count on ruminal material obtained in this manner was $27 \pm 2.23 \times 10^9$ per cc. compared with $93 \pm 19.5 \times 10^9$ per g. for solids and $62 \pm 9.3 \times 10^9$ per cc. for liquid withdrawn from a fistula.

In view of these results, a series of samples was taken by intubation from a number of sheep which were then immediately killed and further representative whole-rumen samples obtained. Counts were made on these for total numbers of microorganisms, the results of which are given in Table 1.

TABLE 1

COMPARISON OF TOTAL COUNT OF MICROORGANISMS IN WHOLE-RUMEN AND STOMACH-TUBE SAMPLES

Sheep No.	727	728	729	730	731	732	X	A5	Mean
Whole-rumen sample	42.8	51.1	61.5	63.5	44.1	45.2	66.5	83.5	59.3
Tube sample	31.5	57.6	53.4	47.7	38.8	29.6	53.9	66.1	49.3

Correlation coefficient of whole-rumen sample on tube sample = 0.84 with 99 per cent. probability.

Per cent. difference between whole-rumen sample and tube sample −17.25 per cent. significantly constant at 99 per cent. probability.

The constancy of the difference and the highly significant correlation between the two series of samples show that the stomach-tube sample reflects accurately the state of the free microorganisms within the rumen, and justifies this method of sampling where comparisons of total counts are being made.

To check the uniformity of samples taken by intubation, series of three samples were taken from five sheep, the tube being withdrawn and reinserted for each sample. In four of the five sheep a high degree of uniformity was obtained. In subsequent work, the agreement between samples from the same sheep under the same dietary conditions, even when taken several days apart (usually on the 7th and 10th day of trial) has been found to be excellent.

(*b*) *Time of Sampling*

Johnson *et al.* (1944) made plate counts in ruminal samples at hourly intervals after feeding. The results obtained by this method showed the bacterial counts to be highest one hour after feeding. Thereafter they rapidly declined. The production of methane followed a similar pattern and was taken to corroborate the evidence of the plate counts.

Direct counts on a series of samples taken at four-hourly intervals from a pen-fed sheep in this laboratory indicated that the maximum total number

was reached between four and eight hours after feeding and was maintained at this level for some hours. The work of Hale, Duncan, and Huffman (1947) on ruminal digestion indicated that the "digestion" of protein and soluble carbohydrates reached a maximum within six hours of feeding, and that the "digestion" of cellulose was approaching its maximum at that time. Very little change was found after 12 to 14 hours. It was considered on the basis of this evidence that the total numbers would be close to a maximum at six hours. All samples were consequently taken six hours after feeding and, with very few exceptions, excellent agreement has been found between samples taken on different days at this time.

III. Experimental Procedure

(*a*) *Design of Experiment*

The feeding trial was designed on the basis of a 5 × 5 latin square in which five sheep, five trials, and five rations of increasing protein intake were used. This layout was adhered to as far as the feeding of the rations was concerned, but it was found that feed refusals so modified the intake of protein that it became necessary to regroup the results according to the actual nitrogen intake.

(*b*) *Experimental Animals*

Five two-year-old Romney Marsh x Merino comeback wethers of similar breeding were selected from available animals on the basis of weight and temperament.

(*c*) *Rations*

The five rations were compounded so that they would be of equal dry matter and crude fibre content, and of similar gross energy value. The main variable in the rations as fed was the crude protein content, which was increased from 3.7 to 18 per cent. in 3.5 per cent. steps. The lowest protein ration was made up of 500 g. of chaffed oaten hay and 173 g. of starch, a total of 673 g. of dry matter fed daily. The protein of the other rations was increased by replacing the starch with an equal weight of casein. The casein in these rations supplied from one-half to four-fifths of the total protein intake. The composition of the diets and their crude protein content are given in Table 2.

All the rations were mixed and weighed into individual paper bags before the commencement of the trial to ensure minimum variation. The rations, together with 6 g. each of sodium chloride and calcium carbonate, were fed daily at 9 a.m.

The chaff of the basal ration had been poorly cut, permitting some selection and scattering and led to some small errors in collecting feed refusals. Some feed refusals were experienced on all rations, causing an overall reduction in protein intake. The actual levels of protein intake became 3, 6.2, 8.7, 9.5, and 12 per cent.

(*d*) *Trial Regime*

Two weeks before the trial all sheep were placed on the basal ration, and the five selected sheep then given the trial rations. In each trial the preliminary feeding period was 14 days, followed by a 10-day collection period in metabolism crates. Where sheep have been continuously on dry feed for several months, 14 days was found to be sufficient to allow adjustment of the balance of the ruminal microorganisms. If, on the other hand, sheep grazing on green material are brought in, a period of at least six weeks is probably required.

TABLE 2
COMPOSITION OF DIETS – DRY BASIS

Diet	Dry Matter (g.)	Oaten Hay (g.)	Starch (g.)	Casein (g.)	Nitrogen (%)	Crude Protein (%)
A	672	499	173	0	0.595	3.72
B	672	499	149	24	1.157	7.23
C	673	499	124	50	1.734	10.84
D	673	499	99	75	2.294	14.34
E	674	499	73	102	2.893	18.08

(*e*) *Collections*

Collections of the urine, faeces, and food residues were made daily at 9 a.m. An aliquot of these materials was taken, bulked, and stored until the end of the trial. The faeces and residue samples were oven-dried daily. Ruminal samples were taken on the fourth, seventh, and ninth days of the collection period and treated as previously described. Counts were made on the two later samples only. The values presented are the mean of these two counts.

(*f*) *Counting Technique*

A Petroff-Hauser bacterial counting chamber, as described by van der Wath (1948), was used in making the counts. After thoroughly shaking the fixed ruminal sample, a final dilution of 1 in 500 was made, using a Thoma pipette and a 0.1 per cent. solution of aniline blue. After thoroughly mixing, the chamber of the counting cell was filled and placed immediately on the stage of the microscope. The preparation was examined, and any showing wide variation or incomplete dispersion was discarded. A dry combination of lenses, 15x oculars and one 60x achromatic objective, was used for all counts, and the number of organisms in 80 small squares counted. The total number always exceeded 200. From the number of organisms counted, the volume of the chamber, and the dilution, the total number per cubic millimetre was calculated.

IV. RESULTS

The data gathered from the trials are presented in Table 3 in the order in which the rations were fed. As can be seen from the results, feed refusals considerably modified the intended protein intakes, and consequently the data

were regrouped on the basis of actual nitrogen intakes. These groups are given in Table 4, together with the nitrogen intake and balance, "true" digestibility of the nitrogen, biological value, and the total number of organisms as determined in the counts.

TABLE 3

NITROGEN BALANCE RESULTS PER 10-DAY PERIOD. BACTERIA PER CU. MM.

Feed	Sheep	Average Body Wt. (lb.)	Dry Matter Intake (g.)	Nitrogen Intake (g.)	Faeces Nitrogen (g.)	"True" Digestibility of Nitrogen* (%)	Urine Nitrogen (g.)	Per cent. Urine Nitrogen of Nitrogen Intake	Nitrogen Balance (g.)	Biological Value* (%)	Microorganisms in Rumen (millions per cu. mm.)
						Trial 1					
A	732	88	5770	29.0	43.13	62	14.82	51	−28.95	80	31
B	730	83	6410	61.0	41.25	91	29.76	49	−10.01	62.5	41.5
C	731	78	6360	98.0	42.46	93	44.77	46	+10.77	62	53
D	728	91	6740	128.0	48.5	91.5	81.7	64	− 2.2	40	61
E	729	87	6350	122.0	44.94	92	74.35	61	+ 3.71	43	61
						Trial 2					
A	731	70	5040	24.5	33.96	76	12.0	49	−21.42	84	28.5
B	728	91	6710	67.2	49.34	96	30.96	46	−13.1	64	40
C	729	86	6730	101.0	43.31	91	43.45	43	+14.25	66	57
D	732	88	6215	87.4	36.9	96	44.29	51	+ 6.21	63	57
E	730	86	6190	112.0	42.47	93	73.87	66	− 4.44	39	55
						Trial 3					
A	728	85	4400	15.3	27.25	82					25
B	729	91	6730	67.4	42.0	93	34.66	51	− 9.26	63	42
C	730	88	6730	101.0	42.82	94	43.2	43	+14.98	66	56
D	731	74	6180	122.9	45.42	91	80.13	65	− 4.20	37	62
E	732	87	4750	77.9	31.88	93	39.26	50	+ 6.8	60	55
						Trial 4					
A	730	86	4780	27.8	33.81	74	14.5	52	−20.57	82	26
B	731	77	5090	50.9	31.87	93					34
C	732	91	5675	73.6	37.44	92	37.53	51	− 1.4	61	56
D	729	95	6660	119.0	47.77	91	75.5	63	− 4.4	41	62
E	728	90	6120	80.1	40.44	92	38.54	48	− 1.12	63	51
						Trial 5					
A	729	91	4740	23.9	33.5	70	13.39	55	−22.9	86	35
B	732	93	5895	60.8	37.1	93	31.4	52	− 7.7	65	42
C	728	94	6505	89.9	43.4	92	45.0	50	+ 1.5	60	55
D	730	87	6720	125.9	49.0	91	75.96	60	+ 1.06	43	62.5
E	731	78	6150	133.0	48.3	89	85.4	64	− 0.7	35	64

* Assuming metabolic nitrogen of faeces = 5.563 mg. per g. dry matter intake and endogenous nitrogen of urine = 1.252 g. per 100 lb. live weight (Hamilton, Robinson, and Johnson 1948).

Figures 1 and 2 show graphically the peculiarity of the behaviour of Group 5.

(*a*) *Nitrogen Balances*

Group 5 was found to have a significantly lower nitrogen balance than Group 4 ($P < 0.01$) but was not different from Group 3. There is a very high correlation between the nitrogen intake and the nitrogen balance for Groups 1 to 4, the regression for the relationship (excluding Group 5) is nitrogen balance $= -35.75 + 0.45\ x$, $r = 0.98$.

TABLE 4
MAIN EXPERIMENTAL RESULTS

Sheep	Nitrogen Intake (g.)	"True" Digestibility of Nitrogen (%)	Per cent. Urine Nitrogen of Nitrogen Intake	Nitrogen Balance (g.)	Biological Value (%)	Microorganisms in Rumen (millions per cu. mm.)
			Group 1			
732	29.0	62	51	−28.95	80	31
730	27.8	74	52	−20.57	82	26
729	23.9	70	55	−22.99	86	35
731	24.5	76	49	−21.42	84	28.5
728	15.3	82				25
			Group 2			
730	61.0	91	49	−10.01	62.5	41.5
728	67.2	96	46	−13.1	64	40
729	67.4	93	51	− 9.26	63	42
732	60.8	93	52	− 7.7	65	42
731	50.9	93				34
			Group 3			
732	87.4	96	51	+ 6.2	63	57
728	89.9	92	50	+ 1.5	60	55
728	80.1	92	48	− 1.12	63	51
732	77.9	93	50	+ 6.8	60	55
732	73.6	92				53
			Group 4			
731	98.0	93	46	+10.77	62	53
729	101.0	91	43	+14.24	66	57
730	101.0	94	43	+14.98	60	56
			Group 5			
731	133.0	89	64	− 0.7	35	64
731	122.9	91	65	− 4.28	37	62
728	128.0	91.5	64	− 2.2	40	61
730	112.0	93	66	− 4.44	39	55
730	125.9	91	60	+ 1.06	43	62.5
729	122.0	92	61	+ 3.71	43	61
729	119.0	91	63	− 4.4	41	62

Figure 2 shows the excessive urinary nitrogen excretion of Group 5. This is significantly different ($P < 0.01$) from Groups 1 to 4 inclusive, and is directly related to the low nitrogen balance and the biological value of the protein in this particular group.

(*b*) *Biological Values*

Biological values were calculated assuming the metabolic faecal nitrogen excretion to be 5.563 mg. of nitrogen per g. of dry matter intake and the endogenous nitrogen of the urine to be 1.252 g. per 100 lb. live weight (Hamilton, Robinson, and Johnson 1948).

The calculated biological values of the protein fed at the five different levels fall into three distinct groups, Group 1 (83), Groups 2, 3, and 4 (63.6, 61.5, and 62.6), and Group 5 (39.7). The high values obtained from Group 1 are due to the low intake and the low digestibility of the nitrogen fed. In Group 5 the low values result from the excessive urinary excretion and are no doubt due to inadequate supplies of energy readily available to the ruminal microorganisms, to the host animal itself, or to both. The low nitrogen balance of Group 5, mentioned earlier, is probably due to the same cause (see Figs. 1 and 2).

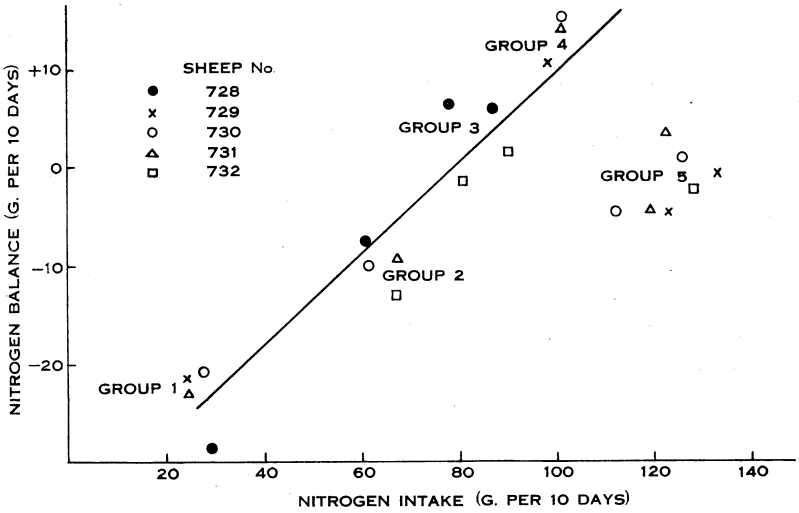

Fig. 1.—Nitrogen balance relationships of the five groups.

(*c*) *"True" Digestibility of Nitrogen*

The "true" digestibility of the nitrogen in Group 1, where the sole source of protein was from oaten hay, is significantly lower than in the other four groups. The average is 70.5 per cent., the range being 62 to 76 per cent. for the four results. The "true" digestibility of the nitrogen in Groups 2 to 5 is practically constant, the overall average being 92 per cent. with a range of 89 to 96 per cent. for the 18 results. In all these rations the oaten hay was supplemented with casein and the very marked effect of this supplementation, particularly in Group 2, is noteworthy.

(*d*) *Counts for Ruminal Microorganisms*

The relationship between the bacterial counts and the nitrogen intakes is shown in Figure 3.

Table 5 gives the individual and average regression equations and correlation coefficients for the data. There is no significant difference between the slopes of the individual regressions. This shows that the sheep all responded in the same manner, and the high average correlation coefficient of 0.98 indicates that most of the increase in numbers can be attributed directly to the nitrogen intake.

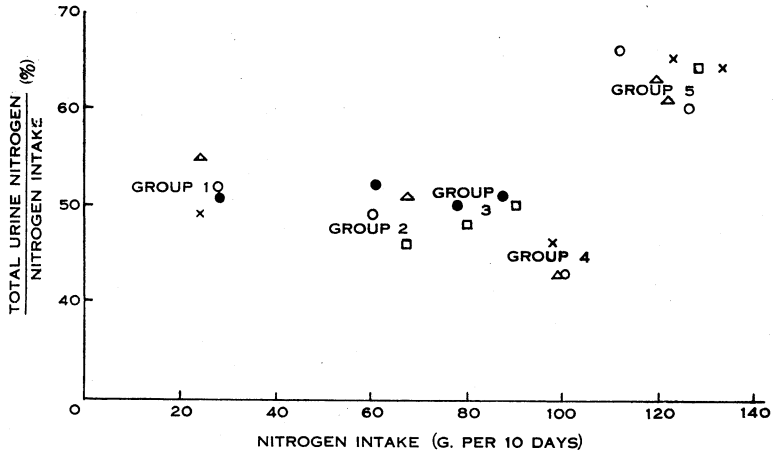

Fig. 2.—Excessive urinary nitrogen excretion of sheep in Group 5.

The variation between individuals is very slight, which is in keeping with the findings of Gall, Stark, and Loosli (1947), and Gall *et al.* (1949), for both sheep and cattle.

TABLE 5

REGRESSION EQUATIONS FOR CORRELATION OF NITROGEN INTAKE ON MICROORGANISMS PER CU. MM.

Sheep	Equation D.F.	Y = a + bX	Correlation Coefficient	Sum of Squares	D.F.
728	4	$Y = 20.82 + 3.36\ X$	0.97	46.241	3
729	4	$Y = 26.3 + 2.9\ X$	0.98	20.806	3
730	4	$Y = 17.6 + 3.58\ X$	0.99	19.217	3
731	4	$Y = 18.44 + 3.47\ X$	0.996	7.912	3
732	4	$Y = 15.3 + 4.76\ X$	0.98	17.086	3
Average equation	20	$Y = 20.95 + 3.43\ X$	0.98	111.262	15

Y = Microorganisms per cu. mm.
X = Nitrogen intake as g. per day.

The numbers of organisms per cubic millimetre ranged from 25 millions on a nitrogen intake of 1.53 g. per day to 64 millions on an intake of 13.3 g. per day. These figures represent largely the "free" organisms rather than the total organisms (which would include organisms attached to plant material) and must be regarded as minima, particularly as samples taken by this method

give lower counts than whole-rumen samples. The magnitude of the counts compares favourably with that found by Gall, Stark, and Loosli (1947), and Gall *et al.* (1949), for winter (non-pasture) ration. The numbers of organisms per cubic millimetre found by these workers, and in the present study, are very much higher than those quoted by Louw and van der Wath (1943), which

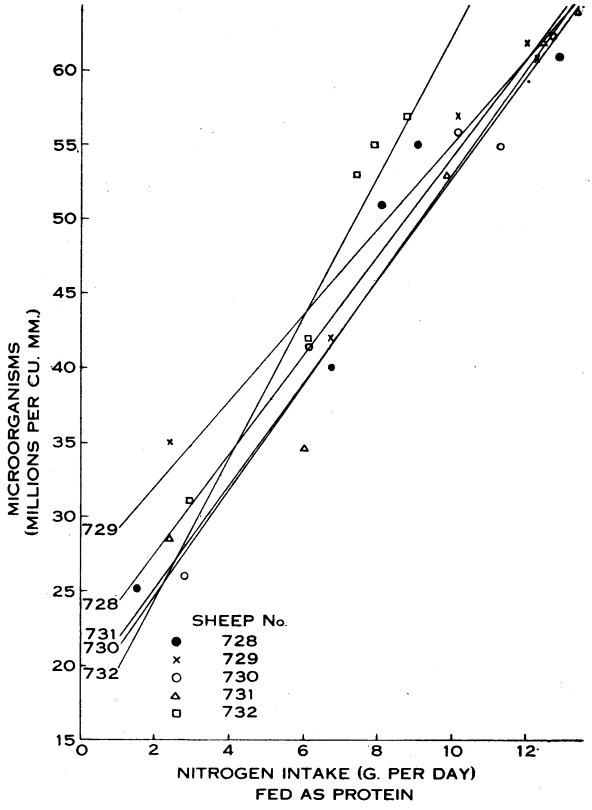

Fig. 3.—Relationship between bacterial count and nitrogen intake for each animal.

range from 1.2 to 1.8 millions (means of groups). This may be due to the lower magnification (Zeiss Huyghen ocular 7x and a Zeiss achromat 40x dry lens) which was used by Louw and van der Wath in their counts.

V. Discussion

The extremely high correlation ($r = +0.98$) found between counts for the total number of free microorganisms in the rumen and the daily intake of nitrogen in the form of protein indicates that, under the conditions of the experiment, the rise in the count from 25 to 64 millions per cu. mm. can be attributed almost entirely to the level of protein ingested. The constant increase in the numbers of the microorganisms with increasing protein intakes, from 3 to 12 per cent. of the dry matter of the rations, suggests that a constant

proportion of the food protein was converted to bacterial protein. The regressions (Fig. 3) show that the fivefold increase in protein intake yielded only a 250 per cent. increase in numbers. This suggests a conversion of food protein to bacterial protein of approximately 50 per cent., which is not greatly dissimilar to the figure (about 40 per cent.) found by McDonald (1948), using an entirely different approach and with a ration in which 82 per cent. of the protein consisted of zein. The conversion was not nearly as complete, therefore, as was inferred by Johnson *et al.* (1942) for protein intakes up to 12 per cent.

It will be noted that the level of protein ingested influenced the numbers of ruminal microorganisms similarly over the whole range of protein intake. In Groups 2, 3, and 4, in which the protein levels were approximately 6, 8.7, and 9.5 per cent. respectively, this is perhaps not unexpected in view of the fact that there were no significant differences in the averages of the groups for the "true" digestibility and biological value of the ingested protein. In each of these three groups the average "true" digestibility was very close to 93 per cent. and the biological value to 63. In Groups 1 and 5, however, there were marked differences which call for further comment.

In Group 1, where the protein intake was only about 3 per cent. and came entirely from the oaten hay, the "true" digestibility of this protein was considerably lower (average 73 per cent.) and the biological value appreciably higher (average 83 per cent.) than in the other groups. These figures are strikingly similar to those given by Smuts and Marais (1942) for poor veld grazing where the protein content was 3 per cent. It is apparent that the starch fed in the present experiment had no appreciable effect on the utilization of the limited quantity of protein available. Nor does it seem likely that the starch had any effect on the numbers of ruminal organisms present. As pointed out in the Introduction, van der Wath (1942) found no increase in the counts when dextrinized starch was added to a basal ration of wheat straw, although further additions of urea or fishmeal raised them considerably. It seems clear, therefore, that in Group 1 the number of microorganisms in the rumen was limited by the amount of available protein and not by any energy considerations.

At the highest level of protein intake (Group 5) the protein was not nearly as efficiently utilized and its biological value fell to an average of only 40. For a casein ration supplying similar amounts of protein Johnson *et al.* (1942) obtained a biological value of approximately 60 but the level of available energy was much higher in their experiments than in these. There seems no doubt that the sheep of Group 5 were in negative energy balance and that additional supplies of energy would have improved the utilization of the protein. On the other hand, the increase in the numbers of ruminal microorganisms from Group 4 to Group 5 was of the same order as the increase from Group 3 to Group 4 and Group 2 to Group 3. It seems very likely, therefore, that the ruminal microorganisms in the sheep of Group 5 were able to satisfy their energy requirements from the available materials and that in

this group, as in the others, the numbers were limited by the protein supply alone. The whole question, however, of the influence of varying intakes of protein and energy on the proliferation of the ruminal organisms is under direct investigation at the present time.

VI. Acknowledgments

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