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Studies on the Alimentary Tract of Merino Sheep in South Africa X.—Notes on the Digestion of Some Sugars in the Rumen of Sheep.*

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In a previous publication from this laboratory (Quin, van der Wath and Myburgh, 1938) in which the subject of ruminant digestion is broadly reviewed, stress is laid upon the great importance of the bacterial degradation of foodstuffs in the rumen as a factor in the conversion of natural products into substances capable of absorption by the animal.

A large part of the natural diet of the sheep is composed of various carbohydrates. While enzymes capable of degrading carbohydrates are elaborated in the more distal portions of the gut, there can be no doubt that very considerate carbohydrate digestion occurs in the rumen and it is indeed probable that this is the main seat of digestion of cellulose, the easily fermented sugars and perhaps to a lesser extent of starch.

It is, therefore, important to study the digestion of various carbohydrates in the rumen with particular reference to the rate of their disappearance and the products obtained.

Without entering into detailed analyses of feeds, it can be stated that the normal diet of the sheep will contain the following substances:—Cellulose, starch, lignin, pentosans, polyuronides (e.g. pectin), sucrose. While sucrose is probably the sugar which occurs naturally in largest quantities, interest also attaches to sugars which can be regarded as probable

* Editorial Note.—Miss McAnally spent some time at Onderstepoort during 1939, as a visiting research worker and started an investigation on digestion in ruminants under the guidance of Dr. J. I. Quin, Head of the Department of Physiology. The outbreak of the war and her departure for England prevented her from continuing with her programme of work. Consequently only the preliminary data obtained by her are considered in this report.

Subsequently investigations of the problem at Onderstepoort necessitated a considerable change both in experimental technique and in the interpretation of the findings.

degradation products of the more complex polysaccharides. Chief among these latter sugars is glucose. Owing to the similarity in their constitution respectively to cellulose and to starch, cellobiose and maltose may be added to the list of sugars under review.

The above considerations led to the formulation of the following programme of work. The rate of fermentation of various sugars and the chief products of their breakdown might first be studied. Following upon this study and in the light of the previous results obtained, the more complex problem of the degradation of polysaccharides might be investigated. The results recorded below represent an attempt to carry out the first part of this programme.

Experimental Technique.

One of the major difficulties associated with a study of digestion in the rumen is the daily variation in the nature of the ruminal contents. Thus an animal which can rapidly dispose of dosed sugar on one day may retain traces of it for some hours on the following day. If, therefore, comparison is to be made between fermentations under various conditions, e.g. of different concentrations of sugar or a series of different sugars, great difficulty will be found in interpreting the results of *in vivo* experiments where there is an uncontrolled variable. Whenever possible, therefore, comparisons were made *in vitro*. Thus rumen ingesta was withdrawn from sheep through a rumen fistula according to the method already described (Quin, v. d. Wath, and Myburgh, 1938). The material could then be divided into aliquots and fermentation thus compared under various conditions by exactly similar ingesta.

There are plainly objections to working *in vitro* which must always be borne in mind when interpreting results and as far as possible must be obviated by controlling experimental conditions. Thus if the ingesta is kept too long *in vitro* acid formation and putrefaction occur thus rendering the conditions unphysiological. Where fermentation is rapid, as in the case of the sugars *in vitro* experimentation was thought to be more reliable. When, however, fermentation was of longer duration, as with cellulose and starch, it was necessary to use another technique which will be described in a later section.

The rate of gas evolution was found to be a useful index of the rate of fermentation of sugars. This was conveniently observed in a series of 5 c.c. graduated fermentation tubes. As it was impossible to add to these the whole ingesta including solid material, a filtrate obtained by squeezing through muslin was used. A portion of the fermentative agent may in this way be lost, but there should be no error in drawing conclusions when experiments are comparative. Further, a filtrate obtained by squeezing through muslin gave no greater rate of fermentation than material which was allowed to run, under gravity, through muslin. The latter, it may be assumed, would contain less solid material than the former. This filtrate, on centrifugalising, gave an opaque centrifugate which showed very little fermentative power. It would thus appear that the fermentative power resides in the fraction of the filtrate which is thrown down on centrifuging.

The time of withdrawal of ingesta for use *in vitro* work was found to be important. The following figures show the gas evolution from a constant weight of glucose by ingesta withdrawn at the times stated:—

Gas evolved in c.c. by 7·5 c.c. of ingesta filtrate acting upon 0·5 c.c. of 20 per cent. glucose solution.

Time in Minutes (after Addition of Glucose when Gas Reading Taken).	Time of Withdrawal of Ingesta.			
	9.0 a.m.	10.15 a.m.	11.30 a.m.	2.0 p.m.
	c.c.	c.c.	c.c.	c.c.
10	0·4	0·35	0·3	0·2
15	1·3	0·5	0·45	0·45
20	—	—	0·6	0·85
25	—	0·7	0·85	1·05
30	3·65	0·95	—	—
45	4·4	1·35	1·4	1·85

The sheep was fed shortly after the 9.0 a.m. ingesta was withdrawn.

The above results, which have been repeatedly confirmed, show clearly that the fermentative power of the pre-feed ingesta is considerably greater than that of material withdrawn later in the day. The investigation of the reason for this difference constitutes a problem in itself which has not yet been solved. For the purpose of the present work, however, these figures make clear that by using the pre-feed ingesta not only will the optimum conditions for *in vitro* work, i.e. short duration of the experiment, be promoted, but also physiological conditions maintained during which foodstuffs are acted upon by the fasting ingesta.

The pH of the contents of the sheep's rumen is maintained at a fairly constant level, around neutrality, by the buffering action of constituents and more especially by the neutralization of the acid which is formed during fermentation by carbonate secreted in the saliva. Addition of buffer solutions to the ingesta filtrate is unsatisfactory since a sufficient quantity to hold the pH will considerably lessen the fermentative power of the ingesta by dilution.

Allowing the reaction to take place in the presence of excess of calcium carbonate is a suitable expedient when it is possible to stir the whole material periodically. In fermentation tube experiments, however, the calcium carbonate cannot be brought in contact with the material in the top of the tube. Its usefulness is therefore insufficient under these circumstances. The natural buffering power of the ingesta is appreciable and should suffice to hold the pH within physiological limits except when large amounts of acid are being formed. It was thus finally decided to use the ingesta without added buffer. It must, however, be borne in mind in interpreting results that acid formation may affect the course of fermentation in its later stages.

When substrate is added to ingesta in solution it is important to keep the proportion of ingesta to added water as high as possible since, in addition to the loss of fermentative power consequent upon dilution, the added water must first be saturated with gas before any gas evolution is observed.

The concentration of sugar relative to ingesta which is optimum for the study of gas formation can be shown to be 0·1-0·2 gm. of any of the sugars investigated per 8 c.c. of ingesta filtrate. This subject is dealt with more fully below when the fermentation of various sugars is discussed.

The conditions for *in vitro* fermentations which have been applied in the ensuing work may thus be summarised: rumen ingesta was withdrawn by aspiration through the fistula before the morning feed from a sheep which received 400 gm. of lucerne hay twice daily, and was dosed 3 litres of water daily in two portions through the rumen fistula. The ingesta was poured on to muslin and the fluid portion squeezed through. Following this 8 c.c. portions of the filtrate were introduced into the fermentation tubes which were then allowed to stand in an incubator at 37°C until their contents had reached this temperature. The substrate was then added in solution in an amount which was never more than 2 c.c. The ingesta and substrate were then mixed by shaking and the fermentation was observed in the incubator at 37°C.

Formation of Gas as a Measure of the Rate of Fermentation of Various Sugars.

On addition of glucose to ingesta filtrate gas formation sets in rapidly, considerable quantities of gas being formed within the first ten to fifteen minutes. Gas formation proceeds until a point is reached, presumably when the supply of sugar is low or exhausted, when the rate of gas evolution falls off abruptly and though gas continues to be produced the rate is much smaller. By observing the gas-evolution in a series of tubes to which different amounts of glucose have been added, it is clearly seen that with increasing glucose content the period of rapid gas evolution is prolonged. The following experiment illustrates this point:—

Gas evolved in c.c. from 8·5 c.c. material containing 7·5 c.c. ingesta filtrate, various volumes of 20 per cent. glucose and the remainder water.

C.c. of 20 Per Cent. Glucose Added.	Incubation Period in Minutes.					
	10	20	30	40	50	60
0·1	0·05	0·05	0·1	0·2	0·3	0·4
0·2	0·2	0·3	0·45	0·65	0·9	1·05
0·3	0·3	1·2	1·8	2·45	2·65	2·8
0·4	0·3	1·2	2·05	2·85	3·15	3·4
0·5	0·25	1·25	2·4	3·1	3·6	3·9
0·6	0·2	1·3	2·4	3·15	3·6	3·85
0·7	·2	1·35	2·45	3·25	3·65	3·85
0·8	0·2	1·35	2·55	3·35	3·7	4·0
0·9	0·2	0·95	2·3	3·05	3·4	3·6
1·0	0·1	1·35	2·65	3·3	3·65	3·8

It appears that while a plentiful supply of sugar is still available, there is a maximum rate of gas evolution which is not exceeded even if the original sugar content is increased in amount. Further, an indication can be seen in the above experiment of a tendency which has been repeatedly demonstrated in a series of similar experiments. If the ratio of weight of glucose to ingesta volume is increased above a value which is approximately 0·2 gm./8 c.c. there is a diminution in the rate of gas evolution.

A comparison was made of the rate of gas evolution from a number of sugars—chief among these were sucrose and maltose, maltose being studied on account of its constitutional similarity to starch. A number of experiments in which these sugars were compared with glucose gave results fully in agreement with that quoted below in which cellobiose as a degradation product of cellulose, and lactose as a sugar wholly foreign to the rumen, were also studied.

Gas evolved in c.c. from 7·5 c.c. ingesta filtrate to which 2·0 c.c. of 8 per cent. sugar was added.

	Incubation Period in Minutes.												
	5	10	15	20	25	30	35	40	45	50	55	60	90
Glucose.....	0·0	0·35	0·75	1·35	1·75	2·05	2·3	2·6	2·8	2·95	3·2	3·4	4·3
Maltose.....	0·0	0·0	0·05	0·1	0·2	0·25	0·3	0·3	0·5	0·6	0·7	0·85	2·15
Sucrose.....	0·0	0·15	0·4	0·65	0·95	1·2	1·5	1·7	1·9	2·1	2·35	2·6	3·5
Cellobiose.....	0·0	0·0	0·0	0·05	0·2	0·2	0·25	0·4	0·45	0·55	0·65	0·7	1·9
Lactose.....	0·0	0·0	0·0	0·0	0·05	0·05	0·05	0·05	0·05	0·1	0·2	0·2	0·9

The relationships between sugars is here clearly shown. Glucose is most readily fermented. The fermentation of sucrose does not start so rapidly as that of glucose but soon attains the same rate of gas evolution. In other experiments the gas evolution from glucose and sucrose was more nearly parallel. While sucrose is a natural constituent contained in many of the food plants consumed by sheep, maltose and cellulose are interesting only by virtue of their constitutional relationship to starch and cellulose. It is not surprising, therefore, that these sugars are much less rapidly attacked by the rumen organisms than is sucrose. Cellobiose is perhaps slightly less readily fermented than maltose but since this was the only experiment where cellobiose was observed the difference cannot be regarded as significant. Other experiments where maltose was compared with glucose also indicate that after an initial period of slow fermentation, gas is evolved from maltose quite as rapidly as it is from glucose at the period of most rapid fermentation. It is possible that the initial lag represents the period of hydrolysis of maltose after which fermentation of the resulting glucose takes place normally. Lactose, as might be expected, is very feebly fermented.

Formation of Acid from Glucose by Rumen Ingesta.

For the further investigation of the breakdown products of sugar in the rumen, glucose was chosen for preliminary study as a simple sugar of fundamental importance.

In order to measure the total acid produced in fermentation it was first necessary to test the efficacy as a method of straight forward titration of the fermentation fluid against alkali using phenol phthalein as indicator. Four portions each of 5 c.c. of ingesta filtrate were taken. One was taken as control and to the others were added one, two and three c.c. respectively of standard $\frac{N}{10}$ acetic acid. Each was then titrated against standard $\frac{N}{10}$ caustic potash using phenol phthalein as indicator. By subtraction it was possible to find the alkali neutralised by the 1st, 2nd and 3rd c.c. of added

acetic acid. Thus it was shown that 85 per cent. of the 1st c.c. is recovered, 85 per cent. of the 2nd and 100 per cent of. the 3rd. It is probable that quantitative titration does not occur until carbonate has been destroyed by addition of sufficient acid to neutralise it. Titration of the acidity was therefore performed after the addition to about 7·5 c.c. of ingesta filtrate of 3 c.c. of $\frac{N}{10}$ H_2SO_4. Further it was shown that 2 minutes aeration after the addition of sulphuric acid reduced the titration value owing to the removal of CO_2. By adopting these two methods it was thus possible to obtain figures for variations in total organic acid content due to fermentation of sugar.

In all cases examined it was found that total acidity rose rapidly to a high level within ten to fifteen minutes after addition of glucose and that further increase of acid was not then appreciable. Comparison of the rate of acid and of gas production showed that gas evolution continues after the acidity has reached its maximum level.

The following two series of results (A and B) are typical and serve to illustrate the above conclusion:—

> Fermentation tubes containing 7·5 c.c. ingesta filtrate plus 0·5 c.c. of 20 per cent. glucose were emptied after the time intervals stated and after the gas volume had been read off. Following this the acid was titrated according to the method described above.

		Time in Minutes.								
		5	10	15	20	25	30	35	40	45
Gas evolved in c.c.	A.	0·3	1·45	3·1	4·25	4·4	4·7	4·95	4·5	4·6
	B.	0·25	0·7	2·8	3·8	4·4	4·6	4·6	3·95	4·2
Acid in c.c. $\frac{N}{10}$ in excess of control.	A.	—	3·45	3·35	3·85	4·05	4·05	3·9	4·0	4·4
	B.	2·1	1·9	3·55	3·65	3·85	3·95	4·45	4·25	4·25

It would thus appear that considerable acid production precedes gas evolution. As an explanation of this fact it may be suggested that oxidation of glucose with the formation of hydrxoy acid of high molecular weight may precede true fermentation with the production of gas and small molecule acids.

In order to test this hypothesis it was necessary to compare the rate of total acid production with that of the volatile and ether soluble non-volatile acids. In all cases the volatile acid content of fermentation fluid was by no means so dependent upon time of incubation as was that of total acids. The curves for volatile acids against time were very irregular in form though there was a general tendency for volatile acid to increase after the first 15-20 minutes.

30 c.c. of ingesta filtrate (A) and 30 c.c. of ingesta filtrate plus 2 c.c. of 20 per cent. glucose (B) were incubated at 37° C. for 15 minutes. Some aluminium sulphate was then added to each and the clear liquid separated from the precipitation protein by centrifuging. The clear liquid together with washings was in each case made up to 100 c.c. 50 c.c. was taken from

each, excess sulphuric acid added and the volatile acids distilled with steam until 50 c.c. of distillate neutralised less than 1·0 c.c. of $\frac{N}{10}$ alkali. The residual liquid was thoroughly extracted with ether and the ether extract titrated with $\frac{N}{10}$ alkali. Following this 25 c.c. was taken from (A) and from (B), $\frac{N}{10}$ alkali was added to neutralise exactly and the material was evaporated to dryness. The resulting salts of organic acids were then taken up in water, the solution filtered and then evaporated to dryness and the residue ignited. The ash will contain carbonates in equivalent amount to the organic acids and their salts which were originally present. By titrating this ash with standard acid, therefore, their amount can be determined.

Cubic Centimetres $\frac{N}{10}$ Alkali Equivalent to:—

	1	2	3	4
Total organic radicals.....(A.)	25·6	19·8	20·4	16·2
Total organic radicals.....(B.)	33·0	28·4	29·0	30·4
Volatile acids.....(A.)	12·9	13·6	16·3	11·9
Volatile acids.....(B.)	9·6	9·0	13·7	12·7
Ether soluble non-volatile acids.....(A.)	1·2	3·7	5·9	3·8
Ether soluble non-volatile acids.....(B.)	1·3	1·4	8·1	3·4

By comparing the (A) and (B) pairs in this table of results it can readily be seen that whereas, in a 15 minute incubation, there is a marked increase in total organic acid radicals, this is accounted for neither by volatile acids nor by ether soluble non-volatile acids.

Further analysis is required to determine the nature of this unidentified acid fraction. Whatever its nature may be it is probable that it represents a stage only in the breakdown of sugar to gas and acids of small molecular size. Demonstration, however, of the existence in the rumen for any considerable length of time of, for instance, hydroxy acids of three, four, five or six carbon atoms would be of considerable interest from the point of view of the nutrition of the animal.

Digestion of Polysaccharides in the Rumen.

Owing to the relative slowness of attack upon polysaccharides such as starch and cellulose by the rumen organisms, the methods described for the study of sugar fermentation are unsuitable.

A successful method has, however, been devised for the study of the disappearance of insoluble materials *in vivo*. This has so far been applied to cellulose only.

Suitable aliquot quantities of cellulose (mashed filter paper) were weighed out accurately on to a series of 2½ inch squares made of some fine natural silk material. The squares were previously moistened to prevent fine particles passing through the pores of the material. The edges were brought together and bound round with silk. About six or eight such bags were lightly bound together and suspended by a silk cord in the rumen of a sheep having a large (5 cm. in diameter) rumen fistula. After suitable periods the whole bunch was removed, a single bag cut off and the rest returned to the rumen.

The residual material in the bag was then thoroughly washed with a jet of water, using the silk as a filter, then dried on a water bath and weighed.

From the few results thus far obtained it would appear that digestion of cellulose, under the circumstances of these experiments does not start until during the course of the second day. In each case it was found that digestion of the cellulose was appreciable between the 20th and 26th hours. Further work is being carried out along these lines.

Summary.

1. Studies were undertaken on the disintegration of various carbohydrates (sugars and cellulose) in the rumen of sheep. For this purpose were utilised adult merino sheep with permanent fistulae in the rumen.

2. With the animals on standardized diets, ruminal ingesta was periodically withdrawn by aspiration through the fistula. This material was then filtered through fine muslin and fermentative activity of the filtrate determined by measuring the volumes of gas evolved in fermentation tubes following the addition of different concentrations of sugars.

3. Results obtained show the extreme rapidity with which sugar is fermented by rumen ingesta, the rate and degree of fermentation depending on (*a*) type of sugar used, (*b*) its concentration in the tubes, and (*c*) the nature of the ingesta and the time of its withdrawal.

4. Accompanying the evolution of gas, there is a rapid rise in total acids within the tubes. This, however, cannot be wholly accounted for either as volatile acids or as ether soluble non-volatile acids, thus necessitating further investigation,

5. Methods are described for determining the rate of disappearance of cellulose within the rumen, through the enclosure of weighed amounts of cellulose in thin silk bags and the suspension of these through the rumen fistula.

Further work along these lines is still in progress.

Acknowledgment.

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