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Studies on the Alimentary Tract of Merino Sheep in South Africa VII.—Fermentation in the Forestomachs of Sheep.

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I. Introduction.

The nutrition of ruminant animals has within recent years developed into a matter of very special significance. This was due primarily to the realization of the economic importance of various products derived from these animals and in consequence of which the world demand for them has steadily increased. As it was further realised that the ruminant body was peculiarly adapted for the transformation of bulky, inexpensive plant material into such valuable products as meat, milk, and wool, a vast programme of research work has been undertaken on the nutritional requirements of various classes of ruminants relative to type and level of production. Moreover the appearance of a wide variety of deficiency states in ruminants acted as a further incentive for more detailed investigations of their nutrition. As a result of all these considerations much knowledge has recently been gained on this particular subject. Based on a wide range of metabolism studies, one of the most important findings in all this work consisted in demonstrating the basic nature and nutritional significance of so-called balanced rations. Much of this information was obtained by the well-known methods of trial and error usually comprised of a correlation between:—

(*a*) the diet fed,

(*b*) the digestibility and utilization of individual nutrients, and

(*c*) body response.

By these methods the significance of the balanced diet, including the relative importance of a wide variety of individual factors, has been clearly established in ruminant nutrition. This has resulted in a decided improvement in production as well as in the successful control of a number of deficiency diseases.

In spite of this newer knowledge, the nutrition of ruminants nevertheless still presents a variety of unsolved problems. This is due, at least in part, to the intricacies of digestion, the physiology of which is less clearly understood in the ruminant than in any of the other domesticated mammals. This, indeed, constitutes an important limiting factor as far as a complete understanding of the nutritional problem in these animals is concerned. In answer to the question why the processes of ruminant digestion should be considered as being more involved than those in other classes af animals, it should be

emphasized that this is due primarily to the presence of the various forestomachs in the digestive system. Seeing that processes whereby food materials are broken down in these compartments are so completely different from those usually associated with the enzyme systems operating in the rest of the digestive tract, it is essential that the peculiarities and relative significance of these two distinct phases of digestion be studied separately. It is to be expected that more detailed investigations along these lines would increase not only our knowledge concerning the basic principles of ruminant nutrition, but at the same time help to elucidate the aetiology of the large variety of digestive disturbances to which ruminants are subject.

In considering the normal conditions present in the forestomachs, it is to be noted that while there is complete absence of digestive secretions from their walls, these organs provide an exceptionally favourable environment for the establishment of a wide variety of bacteria, infusoria, and other micro-organisms which usually contaminate the food supply. It is due largely to the maintenance of optimal conditions in the forestomachs concerning food supply, moisture, temperature, hydrogen ion concentration, oxygen and carbon dioxide tensions, and removal of metabolites, that various groups of micro-organisms are capable of establishing themselves and even of forming such very dense populations in the ruminal mass.

Through the researches of Czepa and Stigler, Wester, Trautmann, Krzywanek, Dukes, Schalk and Amadon, Phillipson, Quin and van der Wath and various other workers, much information has been gained within recent years concerning the physical and mechanical aspects of rumen physiology. Thus the process of regurgitation during rumination and the factors associated with the motility of the forestomachs have been fairly fully elucidated.

In respect to the chemical changes undergone by the food mass in its passage through the forestomachs and the relation that these changes bear to the other phases of digestion, our knowledge is as yet far from complete. It is known, however, that through the presence of various types of bacteria in them, the forestomachs are mainly associated with the disintegration of cellulose and other fibrous food constituents. Likewise acid fermentation of carbohydrates has been established. According to the findings of McElroy and others, the rumen forms the seat of an important vitamin B synthesis. Concerning the significance of the different organisms, Mangold, mainly through the work of Ferber, considers the ruminal infusoria as playing an important role in the biological ennoblement (biologische Veredlung) of plant proteins which, after their transformation into infusorial protein, are rendered more easily available to the animal body. Becker, Schulz and Emmerson on the other hand regard the infusoria merely as commensals and thus without any specific importance in ruminant digestion. Van der Wath, in a recent publication, likewise concludes that ruminal infusoria are not essential, seeing that digestion is well maintained in sheep from which these organisms have been banished. Kleine, in referring to all the micro-organisms in the forestomachs as " Pansen Plankton " (ruminal plancton), considers this as constituting an important item in the diet of the host animal.

While large numbers of micro-organisms are constantly passed out with the ingesta from the forestomachs to undergo disintegration and absorption in the rest of the digestive tract, there are as yet no data available to indicate the exact significance of this in facilitating digestion and thereby of

improving the nutritional state of the animal. All food materials swallowed into the forestomachs are exposed to the action of a densely populated, mixed culture of micro-organisms which has to maintain itself in the face of constant drainage. In contrast to the well-known hydrolytic processes usually associated with digestion, the changes undergone by the food during its passage through the forestomachs are indeed far more complex, seeing that these changes are determined by the constitution and metabolic activity of the microflora present. Accompaying the disintegration of various food materials during this phase of digestion, new compounds are constantly being synthesised in the forestomachs. Little is as yet known about the nature and significance of these products, although the formation of vitamin B complex referred to above serves as an example. Likewise the utilization of such nitrogenous compounds as urea and various ammonium salts in the production of new proteins within the rumen, may be quoted as further evidence of the synthetic powers of the ruminal flora. (Kleine, Schmid and Studt, Harris and Mitchell.)

From these considerations it may be concluded that ruminant nutrition is at least partly dependent upon the products of bacterial metabolism which are rendered available for absorption. As a result of these processes in the forestomachs, the biological value of the food consumed may be altered in such a way as to lead either to its improvement or to its deterioration depending upon the type and density of the microflora. In order to achieve and to maintain optimal conditions within the forestomachs in respect to both the nature of the processes and the speed at which they occur, it is essential, therefore, to stabilize as far as possible this mixed population of micro-organisms by constant satisfaction of the various requirements. In this respect the adequacy or otherwise of their food supply, including that of minerals and other vital factors is of no less importance than that applying to any mixture of micro-organisms under artificial cultivation. A deficiency of any one essential nutrient should, therefore, result in some form of disturbance in the microflora in which either the population as a whole or some of its individual members are subjected to a depression. Should such a disturbance be sufficiently severe, it is obvious that the various processes normally occurring in the forestomachs might be replaced by a series of pathological conditions expressed in symptoms such as atony, stasis, hoven, or diarrhoea.

Concerning the infusorial count in the forestomachs, Ferber in his studies has been able to correlate this with the level of protein intake as well as with the metabolic changes associated with growth and lactation. More recently van der Wath, in a study on bacterial counts in rumen ingesta, demonstrated wide fluctuations in his data depending upon the intake level of proteins and carbohydrates.

From information thusfar available there is evidence to suggest, therefore, that a continued and complete satisfaction of the nutritional demands of the mixed ruminal flora may in itself form an essential prerequisite for the adequate nutrition of the host animal. How a normal flora is constituted, what its food requirements are, and of what specific value it is in ruminant nutrition, all form problems yet to be elucidated. Seeing that fermentation constitutes one of the most characteristic features of ruminal activity, a series of investigations were initiated primarily with the object of studying the nature of the fermentation process, and of the factors influencing it, and finally in correlating if possible, these data with the pathogenesis of bloating.

II. Methods Employed on Fistula Sheep.

Using adult merino sheep with permanent ruminal fistulae, Quin, van der Wath and Myburgh in a previous communication described a method for the continuous recording of the amount of gas generated within the rumen. This was achieved by connecting the rumen through its fistula tube to a large graduated water manometer. By equalizing the water level in the two limbs of the manometer the volume of gas could be read off directly at constant atmospheric pressure. From data collected it was soon evident, however, that there were several drawbacks to this method. Firstly, it was found that the gas volume recorded was subject to a double fluctuation which was due either to a change in the rate of fermentation or to a rise or fall in intra-ruminal pressure coinciding with the movements of the forestomachs. Where the gas volumes were being recorded for periods longer than 10 minutes, the effect of ruminal movements on gas pressure could, however, be clearly differentiated from that due to a change in fermentation rate. A further difficulty arose from the fact that filling of the rumen with rapidly fermentable materials frequently led to frothing up of the ingesta followed by blocking of the fistula tube. As a rule this could be overcome by limiting the amount of test material introduced into the rumen.

Considering the gas production as an index of the fermentation processes in the rumen, an attempt was made in this study to correlate the volume and rate at which gas was generated with various food materials, and with conditions prevailing in the forestomachs.

III. Influence of Diet on Gas Production in the Rumen.

The routine procedure in these tests was to place two or more fistula sheep on a specified diet for several weeks. Animals were kept in separate pens so as to allow for individual feeding and watering. Both food and water consumption were determined daily. During the recording of gas volumes, animals were placed in a specially constructed crush pen, thereby restricting movement during the period in which the manometer was connected to the fistula tube. Provision was made for feeding and watering when required during these periods. Gas volumes were continuously recorded every 5 minutes for 15-30 minutes, usually in the early morning and immediately prior to the consumption of the test meal, the recording being continued for several hours afterwards.

Preliminary observations conducted on sheep in the crush-pen disclosed a frequently repeated eructation of gas through the oesophagus, usually within a few minutes after the consumption of food such as green lucerne. At times this eructation was distinctly audible while at other times it was discernible merely as a shallow retroperistaltic wave in the oesophageal region. This eructation of gas from the rumen was, however, stopped as soon as the manometer was connected to the rumen and a rise in the gas pressure prevented by the maintenance of a constant atmospheric pressure in the manometer. From these observations it was safe to conclude that all " free " gas was finding its way into the manometer instead of escaping under pressure through the oesophagus.

In Table 1 is recorded the results obtained by feeding a variety of test foods either to the same sheep or to different ones during the course of the experiment. In none of the animals examined was there any sign of gas production in the early morning before feeding, the only fluctuation in the manometer readings being that due to the ruminal movements. As shown in

TABLE 1.

Effect of Diet on Gas Production in Rumen.

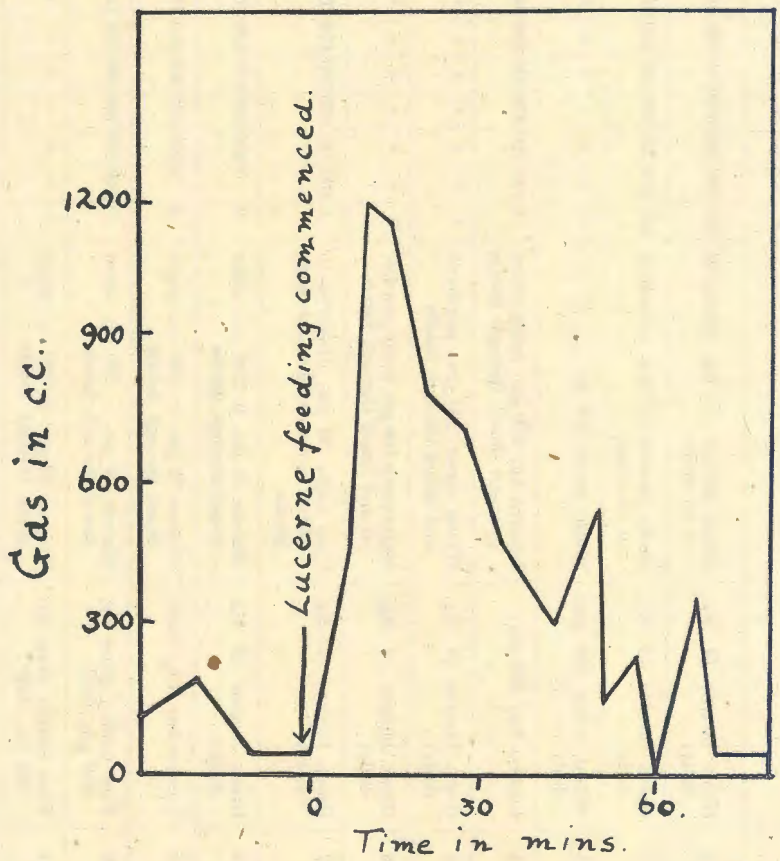
Sheep No.	Basic Ration.	Test Meal.	Gas Production (5 Minute Intervals), in c.c.																		Total Amount of Gas Produced in 90 Mins.
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1	Green lucerne (3 Kg. daily)	Green lucerne 1·5 Kg. consumed in 45 mins.	420	300	520	850	760	640	580	600	600	410	400	480	450	450	340	500	440	325	9,065 c.c.
2	Green lucerne (3 Kg. daily)	Green lucerne 1·5 Kg. consumed in 55 mins.	200	310	350	360	360	350	500	200	360	120	220	140	200	80	120	50	180	50	4,550 c.c.
3	Wheat straw 600 gm. daily	Wheat straw 205 gm.....	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0
4	Lucerne hay 450 gm...	Lucerne hay 200 gm. finely chopped and dosed through fistula	0	140	510	470	500	450	330	260	180	140	140	190	200	170	110	240	300	190	4,520 c.c.
5	Green lucerne (3 Kg. daily)	Wheat straw 200 gm. powdered and dosed through fistula	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0
6	Green lucerne (3 Kg. daily)	Maize samp 100 gm. finely powdered and dosed through fistula	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0
7	Green lucerne (3 Kg. daily)	Cane sugar 50 gm. dissolved in 250 c.c. water and dosed through fistula	0	100	540	550	780	470	350	500	290	390	440	230	60	90	60	40	90	50	5,030 c.c.
8	Green lucerne (3 Kg. daily)	Glucose 50 gm. in 200 c.c. water dosed through fistula	0	0	250	640	670	620	450	420	510	140	40	170	60	100	20	20	0	0	4,110 c.c.
9	Lucerne hay (1 kg. daily)	Glucose 50 gm. in 200 c.c. water dosed through fistula	0	0	260	520	900	800	570	220	130	180	40	10	20	70	40	0	0	0	3,760 c.c.
10	Poor quality grass hay 600 gm. daily	Glucose 50 gm. in 200 c.c. water dosed through fistula	0	240	250	105	100	250	190	190	140	180	200	140	80	80	160	160	20	20	2,505 c.c.
11	Poor quality grass hay 600 gm. daily	Glucose 50 gm. in 200 c.c. water dosed through fistula	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0
12	Poor quality grass hay 600 gm. daily	Glucose 50 gm. in 200 c.c. water dosed through fistula	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0
13	Maize meal 360 gm. and lucerne hay 300 gm. daily	Glucose 50 gm. in 200 c.c. water dosed through fistula *Sheep off feed for past 48 hours.*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0

* No gas produced over period of 90 minutes.

a previous communication (Quin and van der Wath, 1938) this varied from 100 mm. water pressure during the height of ruminal contraction down to 15 mm. below zero during the relaxation phase. The absence of active gas production in the early morning before feeding, affords evidence of the intermittency of the fermentation process, and of its dependence on fresh food in the forestomachs.

Discussion of Results Compiled in Table 1.

1. Sheep kept on a basic diet of lucerne only, which was fed either as green material or as dry hay, were able to cause rapid fermentation of weighed test meals of lucerne consumed in the early morning before routine feeding took place. The amount of gas produced over a period of 90 minutes varied from more than 9 litres (Sheep No. 1) to approximately 4·5 litres (Sheep Nos. 2 and 4). This difference in the total gas yield when identical test meals were fed to individual animals was a feature constantly noted and is to be ascribed solely to the conditions present within the forestomachs. In all these animals, nevertheless, fermentation was of a fulminating character since gas production invariably reached its peak within 30 minutes after the commencement of feeding to be followed by an equally rapid decline (see Curve 1).



Curve No. 1.—Effect of Lucerne feeding on gas production in rumen of sheep.

2. The feeding either of wheat straw (Sheep No. 5) or of maize samp (Sheep No. 6) to animals accustomed to a diet of lucerne, failed to provoke any gas production within 90 minutes. Subsequent gas formation was at best very slow as compared with that noted on lucerne. This indicates that neither cellulose (wheat straw) nor starch (maize samp) is capable of the same rapid fermentation as undergone by certain constituents present in lucerne.

3. The dosing either of cane sugar (Sheep No. 7) or of glucose (Sheep Nos. 8 and 9) in amounts of 50 grams directly into the rumen of animals on a basic diet of lucerne caused prompt fermentation and gas evolution similar to that noted with lucerne itself. As similar fermentation was obtained with juice expressed from fresh green lucerne or with a watery extract from lucerne hay, it was evident that the sugar in the lucerne was the factor responsible for the very rapid fermentation.

4. In animals on a basic ration of poor quality veld grass hay, the introduction of test doses of glucose into the rumen provoked either no fermentation as in sheep Nos. 11 and 12 or a small gas yield of 2·5 litres produced over an extended period and without any sign of a " peak " during the first 30 minutes (sheep No. 10). In this connection it should be noted that sheep No. 10 displayed a particularly strong fermentation on a diet of lucerne and that even on a subsequent ration of poor veld hay its appetite was well maintained. In sheep Nos. 11 and 12, on the other hand glucose fermentation on a lucerne diet was less active while on a subsequent ration of poor veld hay their appetite rapidly declined and with it the power to ferment glucose. Likewise an animal on an adequate ration of maize meal and lucerne hay sheep No. 13), when off its feed for 48 hours, failed to ferment a test dose of glucose. These results indicate that when sheep are either completely starved or kept on a ration consisting of poor quality hay or straw, the power to ferment glucose in the forestomachs is readily suppressed in most animals. In the following section further details will be provided concerning the cause of this change in the fermentation process.

IV. In Vitro Fermentation Tests on Ruminal Ingesta.

A. *Technique.*

In order to obtain more data concerning the fermentation process in the forestomachs, a method was devised whereby small aliquots of freshly drawn ruminal ingesta were fermented *in vitro* and the gas yields determined. By these means several fermentation tests could be carried out simultaneously either on the same or on different samples of ingesta.

The routine procedure was to aspirate 100-300 ml. of the ruminal fluid from the fistula, usually before feeding in the morning when the consistency was more watery than at other times. After straining the material through muslin, amounts of 50 ml. each were poured into Erlenmeyer flasks of 250 ml. capacity and placed in a water bath the temperature of which was controlled at 39° C. Repeated readings previously carried out on the temperature in the rumen of fistula sheep were found to fluctuate round about 39° C. A moveable tray, operated by a small motor and capable of holding the flasks, was fitted inside the water bath thereby ensuring a constant to and fro movement of the material. The object of this was to imitate the mixing of the ingesta by the normal ruminal movements and to promote the escape of

gas from the fermenting mass. A suitable air-tight connection of each flask to an individual water manometer mounted on a wooden frame, allowed for direct readings to be taken on the gas volume at atmospheric pressure.

After a series of preliminary tests on the fermenting qualities of ruminal fluid, the standard method finally adopted consisted in the measurement of the rate of gas production recorded from one flask every 10 minutes over a period of 30 minutes immediately after the addition of 1 ml. 20 per cent. glucose solution (final concentration of 1 in 250 in ruminal fluid). This flask acted as control in a complement of 4 samples which as a rule were fermented simultaneously. Prior to the addition of the test material, the gas yield from the untreated ruminal fluid in each flask was likewise measured over one or more periods of 10 minutes each.

B. *Glucose Fermentation by Ruminal Ingesta.*

In a previous section evidence was submitted of the rapid fermentation undergone by glucose when introduced into the rumen of well-fed sheep as compared to ones on a poor diet. The same animals were used for supplying ruminal material in conducting the *in vitro* fermentation tests.

The following Table, 2, indicates the results obtained when 50 ml. amounts of ruminal fluid drawn from the same sheep on separate days, were fermented with 1 ml. 20 per cent. glucose, while the diet was kept constant at 1 kilogram lucerne hay daily.

TABLE 2.

Date.	Gas produced before adding Glucose (10 Minutes Period).	Gas produced after adding 1 ml., 20 Per cent. Glucose.			Total Gas production during 30 Minutes.
		1st—10 mins.	2nd—10 mins.	3rd—10 mins.	
1.....	2·0 ml.	11·7	7·3	4·7	23·7
2.....	0·9	8·8	8·2	2·8	19·8
3.....	1·0	16·1	5·9	1·9	23·9

From the above table it will be noted that while there is practically no liberation of gas from the ruminal fluid drawn before feeding in the early morning, the addition of glucose to it provokes prompt fermentation and gas production which reaches its maximum within the first 10 minutes. During the following 20 minutes it rapidly reverts to a level only slightly higher than that noted before the addition of sugar. Moreover, there is a close correlation in the total amount of gas produced from ingesta collected on different dates. With constant shaking of the fluid during fermentation the total gas yield was found to fluctuate between 20 and 25 ml. in 30 minutes. In the absence of shaking movements on the other hand, the yield of gas decreased to less than half over the same period while the rate of fermentation became more prolonged. (Curve 2.)

Table 3 presents the results obtained in a comparative fermentation test using ruminal ingesta drawn before feeding from eight different fistula sheep. All animals were kept on a daily diet of 1 kilogram lucerne hay.

TABLE 3.

Glucose Fermentation in Ruminal Fluid from Different Sheep.

Sheep No.	Amount of Gas produced (ml.) before addition of Glucose (10 Minutes Period).	Amount of Gas produced after addition of Glucose.			Total Gas Production (30 Minutes).
		1st Period (10 Minutes).	2nd Period (10 Minutes).	3rd Period (10 Minutes).	
1	1·2	12·5	9·7	5·3	27·5
2	0·8	10·0	7·1	3·3	20·4
3	1·3	13·5	6·2	1·9	21·6
4	1·0	11·7	8·6	4·7	25·0
5	1·3	13·3	8·1	3·1	24·5
6	1·1	7·2	5·3	3·3	15·8
7	0·7	16·1	7·7	1·8	25·6
8	0·8	13·0	9·4	2·8	25·2

From the above results it can be concluded that with eight sheep kept on the same diet of lucerne hay, samples of their ruminal fluid show a close similarity in their power to ferment glucose, except in the case of sheep No. 6 in which the gas production is somewhat lower than in the other animals.

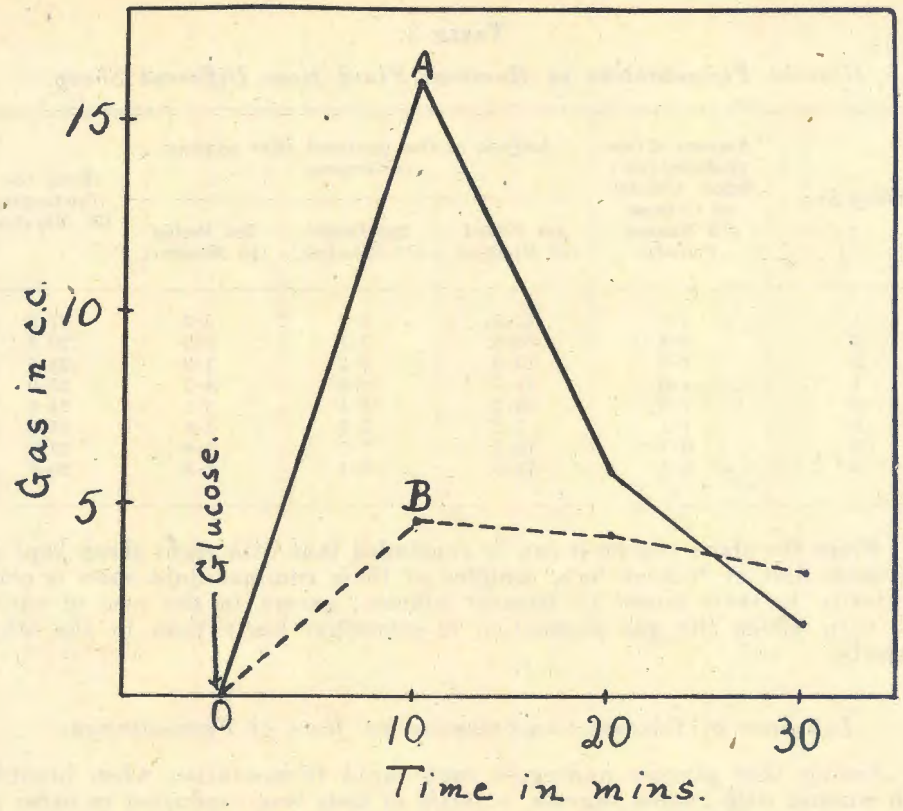
Influence of Glucose Concentration on Rate of Fermentation.

Seeing that glucose undergoes such rapid fermentation when brought into contact with rumen ingesta, a series of tests was conducted in order to ascertain how the rate of fermentation was influenced by varying the concentration of glucose present. This is illustrated in Table 4, in which rumen material from the same animal was used throughout the various fermentation tests.

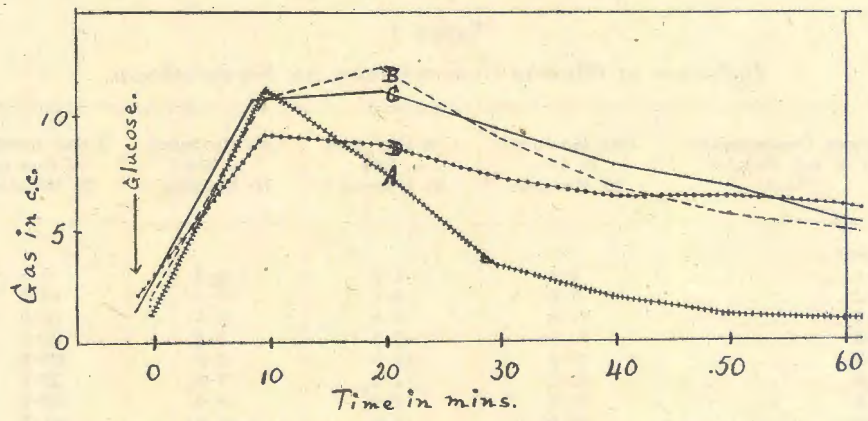
TABLE 4.

Influence of Glucose Concentration on Fermentation.

Glucose Concentration in 50 ml. Ruminal Fluid.	Gas Produced in 1st 10 Minutes.	Gas Produced in 2nd 10 Minutes.	Gas Produced in 3rd 10 Minutes.	Total Amount of Gas in 30 Minutes.
Per cent.—				
0·1	4·4	1·2	0·2	5·8
0·2	7·0	3·1	0·5	10·6
0·3	11·3	3·8	0·5	15·6
0·4	12·7	7·2	1·1	21·0
0·8	12·5	11·7	5·6	29·8
1·2	12·2	12·8	7·8	32·2
1·6	12·0	12·5	8·0	32·5
5·0	10·9	12·0	9·0	31·9
10·0	10·6	10·9	8·7	30·2
15·0	9·1	8·6	7·2	24·9



CURVE No. 2.—Effect of shaking on glucose fermentation by rumen ingesta.
A=shaken. B=unshaken.



CURVE No. 3.—Influence of glucose concentration on fermentation by rumen ingesta.
A=0·4 per cent. glucose. B=5·0 per cent. glucose. C=10·0 per cent. glucose. D=15·0 per cent. glucose.

Data in the above table indicate that fermentation as judged by gas production, is closely related to the concentration of glucose up to a level of 0·4 per cent. With amounts greater than this the fermentation level taken over a 30 minute period, remains practically constant until a concentration of 15 per cent. glucose is reached when there are definite signs of depression in the fermentation rate. (Curve 3.)

Repeated addition of glucose at 30 minute intervals to the same rumen material was found to result in a rapid decline in the gas production which was clearly noticeable even when the second addition of glucose was made. This decline was characterized mainly by the disappearance of the initial high "peak" which was replaced by a lower and more even level of gas production when further amounts of glucose were added.

Likewise freshly drawn rumen material was found to lose its power of fermenting glucose on being continuously shaken for 3 hours in the warm water bath or when allowed to stand either at room temperature or at 0° C. for 24 hours. The addition either of glucose, molasses or of $\frac{N}{10}$ HCl to fresh rumen fluid did, however, preserve the fermenting quality of the fluid when this was subsequently tested with glucose after 24 hours standing. In Table 5 data are presented which illustrate this finding:—

TABLE 5.

Influence of Sugars and of Acid in Preserving the Fermenting Quality of Ruminal fluid.

Material added to 50 ml. fresh rumen fluid.	Total amount of gas produced when ingesta subsequently fermented with glucose after standing at room temperature for 24 hours.
1. Control flask	1·3 ml. gas.
2. Glucose 0·2 gram	20·5
3. Glucose 1·0 gram	0·7
4. Molasses 0·5 gram	21·7
5. Molasses 2·5 gram	Nil.
6. $\frac{N}{10}$ HCl 5 ml	11·9
7. $\frac{N}{10}$ HCl 10 ml	18·9
8. $\frac{N}{10}$ HCl 15 ml	19·0

From this table it is noted that whereas small amounts either of glucose or of molasses aid in preserving the fermenting power of ruminal fluid, larger concentrations definitely inhibit it. The addition of 15 ml. $\frac{N}{10}$ HCl likewise allowed normal glucose fermentation to take place 24 hours after the material had been drawn.

C. *Fermentability of Different Carbohydrates.*

In addition to the fermentation of glucose *in vitro*, a series of comparative tests was carried out on various other sugars and also on starch. The rumen ingesta used in these experiments was derived throughout from the same animal, while a control glucose fermentation test was included in every batch of samples fermented.

TABLE 6.

Fermentation of Different Carbohydrates by Rumen Ingesta (50 ml.).

Test Material.	Gas Formed 1st 10 Minutes.	Gas Formed 2nd 10 Minutes.	Gas Formed 3rd 10 Minutes.	Total Amount Gas formed in 30 Minutes.
	Ml.	Ml.	Ml.	Ml.
Glucose 0·2 gram	13·7	6·2	2·3	22·2
Fructose 0·2 gram	12·3	8·2	1·9	22·4
Sucrose 0·2 gram	11·0	4·0	1·8	16·8
Maltose 0·2 gram	3·1	3·1	3·2	9·4
Mannite 0·2 gram	2·6	2·8	3·2	8·6
Lactose 0·2 gram	2·2	1·6	1·3	5·1
Galactose 0·2 gram	2·1	1·0	1·2	4·3
Arabinose 0·2 gram	1·6	1·0	1·0	3·6
Xylose 0·2 gram	1·6	0·9	0·7	3·2
Rhamnose 0·2 gram	1·4	0·8	0·7	2·9
Raw starch (tapioca) 0·2 gram	1·1	0·9	0·4	2·4
Boiled starch (tapioca) 0·2 gram	1·0	1·0	0·7	2·7

The results in the above table indicate that while both glucose and fructose undergo an extremely rapid and identical type of fermentation, that of sucrose (cane sugar) is somewhat slower although still exhibiting a definite peak period within the first ten minutes. Maltose and mannite on the other hand are both fermented at a much slower speed, fermentation being prolonged and without evidence of any peak period within the first 30 minutes. Fermentation of both lactose and galactose is very feeble while that of arabinose, xylose and rhamnose is even less evident. Likewise starch, either raw or boiled, shows no signs of undergoing fermentative disintegration within 30 minutes. From the first hour onwards there is, however, a notable difference in the fermentation rate of the two starch samples. Thus soluble starch produced a total of 24·5 ml. gas within 4 hours as against a yield of 8·5 ml. only by the raw starch.

D. *Effect of Starvation on the Activity of the Ruminal Fluid.*

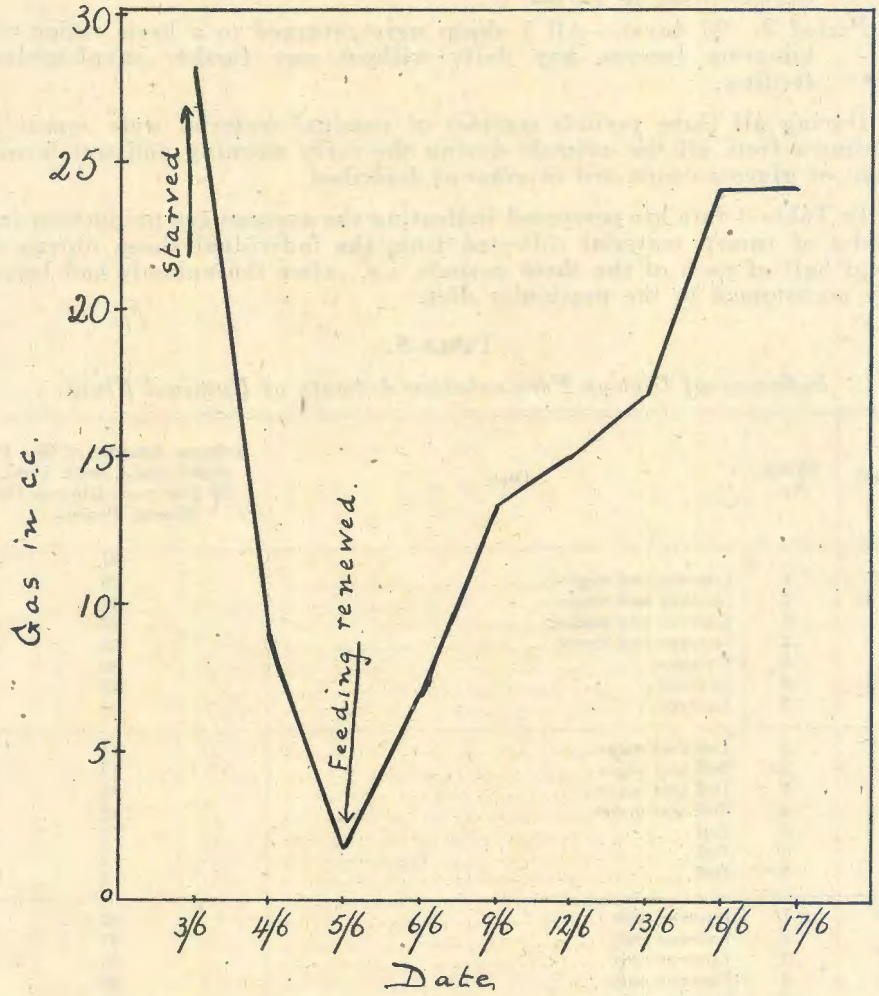
As indicated previously, starvation of a sheep for 48 hours resulted in a marked depression in the fermentation of glucose when dosed into the rumen. This observation was subsequently further investigated *in vitro* on rumen material withdrawn periodically from a fistula sheep undergoing total starvation and kept from water. The results of this test are set out in Table 7. The quantities of rumen material and glucose used were the same as those previously recorded.

TABLE 7.

Activity of Ruminal Fluid during Starvation.

Period of Starvation.	Amount of Gas Produced.			Total for 30 Minutes.
	1st 10 Minutes.	2nd 10 Minutes.	3rd 10 Minutes.	
17 hours	16·5 ml.	8·5	3·0	28·0 ml.
41 „	4·5	2·7	1·8	9·0 „
65 „	1·3	0·2	0·2	1·7 „

From this test it is evident that starvation causes a rapid and practically complete suppression in the fermenting power of ruminal ingesta. This is accompanied by increased wateriness of the material and the absence of any froth as usually noted in the ingesta of the normally fed animal. By restoring the animal to its full diet after having been starved for 65 hours, it was found that the normal daily consumption was reached only after the third day. Glucose fermentation on the other hand was even more profoundly influenced by starvation since the normal gas production was not restored until the tenth day after feeding had been resumed. (See Curve 4.) Results similar to the above were obtained in a subsequent experiment in which 5 sheep were starved for a period of 3 full days.



CURVE No. 4.—Effect of starvation of sheep on glucose fermentation by rumen ingesta.

E. *Type of Diet in Relation to the Character of the Ruminal Fluid.*

Using 7 fistula sheep, a long-term experiment was conducted with the object of ascertaining to what extent the ruminal fluid, as measured by its glucose fermenting properties, was influenced by various types of diet. This was divided into three distinct periods as follows:—

Period 1. (45 days).—All 7 sheep were on a basic ration of 1 kilogram lucerne hay daily. In addition sheep Nos. 1 and 2 received 100 grams cane sugar daily, while sheep Nos. 3 and 4 were each fed 200 grams crushed yellow maize daily.

Period 2. (97 days).—All 7 sheep were on a basic ration of 600-800 grams teff hay, the supplementary ration of sugar and maize being continued as in period 1.

Period 3. (60 days).—All 7 sheep were returned to a basic ration of 1 kilogram lucerne hay daily without any further supplementary feeding.

During all three periods samples of ruminal material were repeatedly withdrawn from all the animals during the early morning and test fermentations of glucose conducted *in vitro* as described.

In Table 8 data are presented indicating the average gas production from samples of rumen material collected from the individual sheep during the second half of each of the three periods, i.e., after the animals had become fully accustomed to the particular diet.

TABLE 8.

Influence of Diet on Fermentative Activity of Ruminal Fluid.

Period.	Sheep No.	Diet.	Average Amount of Gas Produsced (ml.), with 1 ml., 20 Per cent. Glucose (30 Minute Period).
			Ml.
1	1	Lucerne and sugar	29
	2	Lucerne and sugar	36
	3	Lucerne and maize	26
	4	Lucerne and maize	35
	5	Lucerne	30
	6	Lucerne	28
	7	Lucerne	31
2	1	Teff and sugar	14
	2	Teff and sugar	17
	3	Teff and maize	10
	4	Teff and maize	12
	5	Teff	6
	6	Teff	6
	7	Teff	9
3	1	Lucerne only	22
	2	Lucerne only	27
	3	Lucerne only	29
	4	Lucerne only	26
	5	Lucerne only	29
	6	Lucerne only	26
	7	Lucerne only	27

These findings indicate that the supplementary feeding either of cane sugar or of starch in the form of crushed maize produces little if any change in the activity of the ruminal fluid while animals are kept on a basic ration of lucerne hay. When, however, teff hay is substituted in place of lucerne, there follows a decided alteration of the ruminal ingesta in that its power to ferment glucose is severely depressed. This is especially marked in two out of three control animals (Nos. 5-7) in which gas production becomes very low.

V. The Nature of the Enzyme System in Ruminal Ingesta Responsible for Fermentation of Sugars.

Following the demonstration of the rapidity with which glucose, fructose and sucrose are fermented by ruminal fluid as expressed in the volume of gas liberated, an attempt was made to locate and identify the factor responsible for this process. For this purpose freshly drawn and strained ruminal fluid was used. Microscopically this revealed the presence of a wide variety of micro-organisms including several species of infusoria, many species of bacteria, pseudo-yeast cells, and mould spores. An account of some of these organisms and their respective activities is presented in publications by Baker and Martin, and more recently by van der Wath. On a diet consisting exclusively of lucerne hay this fluid assumed a densely turbid yellowish green colour. On standing, a finely flocculent green precipitate settled out within 30 minutes leaving a less turbid yellowish grey supernatant fluid. The precipitate was found to be comprised largely of chlorophyll which formed a thick colloidal mass.

Tests carried out on this supernatant fluid showed that the fermentation of glucose was as active as that in the original material, thereby indicating that the enzyme system was not adsorbed by the colloidal chlorophyll which could be discarded after its precipitation. Most of the infusoria, moreover, settled out with the chlorophyll and frequently formed a distinct, milky-white layer at the bottom of the flask. The remaining infusoria, mostly of the smaller varieties, could be readily thrown down by slow speed centrifugation ($\pm$500 r.p.m.) within one minute. As the supernatant fluid still retained full activity, this afforded evidence that the infusoria were not associated with this process of rapid sugar fermentation. By centrifugation of supernatant fluid for variable periods further aliquot samples of fluid were obtained for the fermentation tests. In this case centrifugation was carried out at a speed of 3,000 r.p.m. which resulted in the progressive precipitation of a thick, greyish, white slimy mass and a decrease in the turbidity of the supernatant fluid.

Table 9.

Gas Production (ml.) from Supernatant Ruminal Fluid (50 ml.) Centrifuged for Variable Periods. 1 ml. 20 per cent. Glucose subsequently added.

Duration of Fermentation.	Duration of Centrifugation (3,000 r.p.m.).				
	Normal Material.	1 Minute.	2½ Minutes.	5 Minutes.	30 Minutes.
10 minutes.....	15·5	7·8	5·6	4·8	3·0
20 minutes.....	9·0	3·2	1·9	1·5	1·4
30 minutes.....	3·0	0·9	0·7	0·5	0·9
Total gas in 30 minutes....	27·5	11·9	8·2	6·8	5·3

These results indicate that at a speed of 3,000 r.p.m. the activity of the supernatant fluid is promptly reduced from a value of 27·5 ml gas to 11·9 ml. within one minute of centrifugation. This is followed by a slowly progressive decrease to 5·3 ml. after 30 minutes. From the ease with which the main part of the active agent could be precipitated, it was concluded that the rapid fermentation of glucose thusfar recorded both in the rumen and *in vitro* tests, was associated not with a free enzyme system but with cellular elements of relatively large size. This was confirmed by microscopic examination of the precipitate which was found to be comprised of a dense mass of clear oval-shaped cellular organisms with an average size of $8 \times 4\mu$. On the other hand the number of bacteria, comprised mostly of small rods and cocci, showed no significantly greater concentration in the precipitate than in the supernatant fluid, due evidently to insufficient centrifugation. By suspending these oval shaped cells, after being washed in water three times, in a solution of 0·2 per cent. sodium bicarbonate, it was found that the addition of glucose was followed by rapid gas production similar to that noted with the untreated ruminal fluid. Thus it could be shown that the precipitate from 50 ml. ruminal fluid when suspended in 50 c.c. 0·2 per cent. $NaHCO_3$ to which 1 ml. of 20 per cent. glucose was added produced 25 ml. of gas with 30 minutes.

The staining of small quantities of the material with equal amounts of Gram's iodine solution, both before and after the addition of glucose, resulted in a rapidly developing brown discoloration which became noticeable within 5 minutes following the addition of glucose. When stained with iodine solution after standing in the water bath for 30 minutes at 39° C., the material changed to a dark chocolate brown colour with a tendency towards rapid sedimentation of the darkened particles. Microscopically this proved to be the oval cells described above. They appeared to be distended with a homogeneous dark brown material when stained after the addition of glucose. In contrast they were of a clear hyaline appearance in the absence of sugar. From this staining reaction and also from subsequent chemical analysis it was evident that these cells were capable of rapidly transforming glucose into glycogen which was stored for variable periods within their cytoplasm. Moreover, this synthesis of glycogen was associated with a fulminating type of gas production, the peak period of which was reached within ten minutes after the addition of glucose. This was followed by a distinct " break " in the gas curve, marked by the onset of a second phase, during which there was a prolonged evolution of minute amounts of gas only. Similar organisms were found in the ruminal ingesta of all sheep on an adequate diet, although there was evidence of wide variation in their numbers in the different animals. Likewise the type of diet was found to exert an important influence, as larger concentrations of these organisms were noted with lucerne feeding than on a ration comprised of poor quality grass hay, the feeding of which resulted in a rapid dwindling down of their numbers.

From its structure and general behaviour this organism was clearly related to the yeasts. It could, however, be differentiated from true yeasts (Saccharomyces species) in that multiplication took place by binary fission after the appearance of a faint cleavage line across the centre. This was followed by the liberation of two identically shaped daughter cells instead of the typical bud formation as seen in true yeasts. In regard to its morphological characteristics it showed a very close resemblance to the false yeast, *Schizosaccharomyces octosporus* as described by Beyerinck. Accordingly this

species encountered in the rumen ingesta of sheep was named *Schizosaccharomyces ovis*. (See Figure 1.) Similarly shaped yeast cells although of larger size were found in the ruminal ingesta of cattle. Whether this constitutes a distinct species from that seen in sheep remains to be decided.

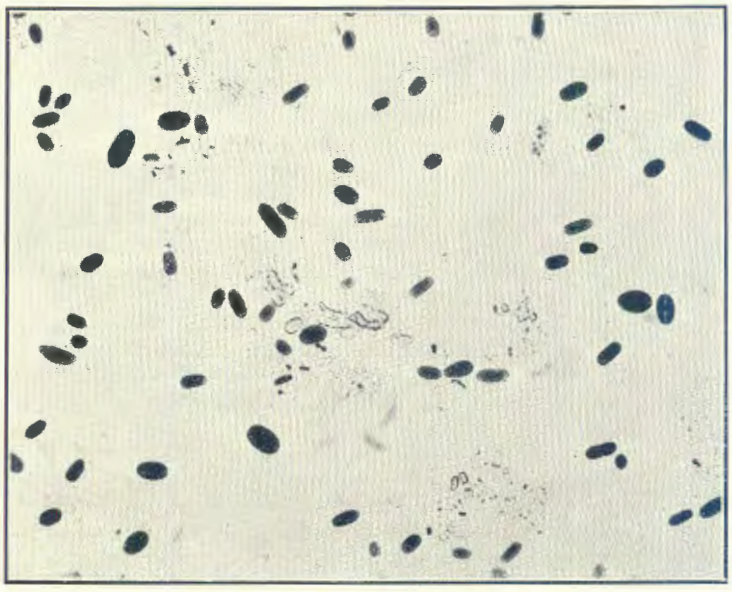


FIG. 1.—Pseudo yeast cells (Schizosaccharomyces ovis) from rumen of sheep. Stained with Grams iodine soln. to show glycogen content of cells. Enlargement 640×.

A. *Influence of Starvation on Yeast Cells in Ruminal Ingesta.*

In this experiment five sheep with rumen fistulae were kept on a daily diet comprised of 1 kilogram dried lucerne hay per sheep. Repeated fermentation of glucose added to fresh samples of rumen ingesta revealed the characteristic fulminating type of gas development which after reaching its peak within the first ten minutes, rapidly subsided in the following twenty minutes. In every instance this was associated with a definite synthesis of glycogen in the yeast cells, large numbers of which were present in the ingesta of all five sheep. Added to the formation of glycogen by the yeast cells, there was evidence of an equally rapid synthesis of polysaccharide (probably starch) from glucose by various species of iodophilic cocci and small rods resembling those described by Baker and Martin. This was revealed by the intense blue staining of these organisms with iodine solution in contrast to the dark brown colour assumed by the yeast cells. The extent to which starch was synthesised, judging by the starch-iodine reaction, was, however, completely overshadowed by the numerous yeast cells, heavily laden with glycogen.

Following the above observations, all animals were completely starved for a period of three days after which they were returned to their normal diet of lucerne. Meanwhile, fermentations and microscopic examinations

were carried out daily on ingesta collected from each animal. After starvation for 24 hours there was a decided decrease in the gas yield from all the ruminal samples which coincided with a drop in the number of yeast cells and the amount of glycogen formed. On the second, and even more so, on the third day of starvation, only minute traces of gas were produced from the rumen material while the number of yeast cells and with it the amount of glycogen formed also showed a very sharp decrease. Following the return to their lucerne diet, 4 out of 5 animals consumed only half their daily ration of 1 kilogram on the first day. By this time yeast cells had completely disappeared from the rumen ingesta of all sheep. On the fourth day all animals were still consuming less than their usual ration. There was, however, evidence of increased gas production from glucose although yeast cells, and with them glycogen formation, still remained absent. Instead there appeared vast numbers of iodophilic cocci which showed a rapid synthesis of starch from the glucose added. Moreover, the gas curve, instead of the usual fulminating type, lacked its high peak period during the first ten minutes. In this it was replaced by a slow rate of gas evolution which was maintained at a practically even level after the 30 minute test period. It was on the sixth day only, following the end of starvation, that all animals consumed their full ration. At the same time a few yeast cells were just reappearing in the ingesta from two sheep. Gas production remained slow although at a slightly higher level than on the previous days. This was associated with intense starch synthesis from the test glucose by the greatly increased numbers of iodophilic cocci which appeared to be firmly established in the rumen. In 4 out of the 5 sheep yeast cells apeared fully re-established in the ruminal ingesta only after a period of 14 days. This was accompanied by the return of extensive glycogen synthesis from glucose and a more rapid rate of gas production. On the other hand the synthesis of starch from sugar by the iodophilic micro-organisms was effectively suppressed in the presence of increasing numbers of yeast cells. These observations afforded very striking evidence of the keen competition displayed by the different ruminal organisms for sugar and also of the manner in which it was utilized by the various groups. Moreover, they demonstrated that the fermentation of sugars by ruminal organisms is associated both with the production of variable amounts of gas as well as with the synthesis of new carbohydrate which is stored within their cytoplasm. In sheep on a diet of lucerne hay the most prominent organism in this respect is the species of false yeast described above. Through its presence, part of the glucose added to fresh ruminal ingesta, when shaken at 39° C. in the presence of air, undergoes extremely rapid oxidation with the production of large volumes of gas. Simultaneously with this, another fraction of the glucose is actively assimilated by the yeast cells and synthesised into glycogen which is stored as reserve carbohydrate. Following suppression of this yeast strain through starvation of the host animal, the yeast cells are readily supplanted by a dense culture of strongly iodophilic cocci as soon as feeding is resumed. These organisms are responsible for an active synthesis of starch from glucose rather than that of glycogen as noted in the yeast cells. Gas production also is at a lower level although maintained for relatively longer periods. With the subsequent re-establishment of the yeast population in the rumen there follows a regression in the synthesis of starch from sugar by the iodophilic microflora.

Concerning the significance of this carbohydrate synthesis by ruminal organisms either as a source of energy or in the synthesis of vitamin B and other compounds, further research work is to be undertaken.

B. *Carbohydrate Assimilation in Other Organisms.*

From investigations on the colourless alga, *Prototheca Zopfii*, Barker was able to demonstrate a rapidly occurring assimilation of such organic compounds as glycerol, ethyl alcohol, glucose, acetic-, propionic-, butyric-acids and valeric acid by cell suspensions of the alga. This process of oxidative assimilation as it was termed, coincided with the consumption of oxygen and the production of carbon dioxide in amounts varying with the nature of the substrate. During the primary phase of the reaction the substrate was rapidly converted through oxidation into a carbohydrate and stored as glycogen within the cells. This was followed by a slowly proceeding secondary phase during which glycogen breakdown became associated in the processes of cell synthesis. Depending on the substrate, from 50 to 80 per cent. of its carbon was assimilated in this manner.

Results essentially similar to those reported by Barker were obtained by Clifton and Logan in studies on washed suspensions of *Escherichia coli*. Thus oxidation of such substrates as acetate, lactate, propionate, glycerol and glucose instead of being carried to completion resulted in the assimilation of a portion of the substrate as carbohydrate by the cells. Moreover, there was evidence that the processes of dissimilation (respiration) and assimilation were closely connected and probably of the nature of coupled reactions. As the amount of oxygen consumed during the primary phase of the reaction always forms a constant proportion of the total amount required for oxidation of a particular substrate it was concluded that the ratio of synthesis to oxidation remained constant for any given system. In the presence of suitable concentrations of sodium azide (NaN_3) or of alpha dinitrophenol, assimilation was inhibited while the oxidation of the substrate was allowed to proceed to completion. A similar type of poisoning was caused by monoiodoacetate which according to the findings of Brücke inhibited the formation of glycogen by yeast both from glucose and ethyl alcohol. Likewise, Clifton found that oxidative assimilation by *Pseudomonas Calco-acetica* and *Escherichia coli* was prevented by iodoacetate.

According to the findings of Winzler and Baumberger a large percentage of glucose which disappeared from a yeast suspension in the presence of oxygen was synthesised to intracellular carbohydrate. In this manner three-fourths of the glucose was stored while the remaining one-quarter was oxidised. Likewise 58 per cent. sodium acetate was oxidised while the remaining 41 per cent. was synthesised. In the absence of air as in alcoholic fermentation on the other hand, 70 per cent. was fermented while only 30 per cent. became stored by the yeast cells. There is, however, as yet no unanimity concerning the various phases of sugar fermentation. Thus Willstätter and Rohdewald claim that the synthesis of polysaccharides, mainly as glycogen, forms an essential stage in the fermentation of glucose and maltose prior to the process of phosphorylation. Kruyk and Klingsmuller, on the other hand, noted a rapid disappearance of glucose from yeast suspensions without corresponding synthesis of any glycogen. According to the findings of Goda, addition of sugar to fresh beer yeast results in a brief induction period during which glycogen accumulates within the cells. In older cultures, however, no such glycogen formation could be demonstrated. In experiments on pure cultures of *Saccharomyces cerevisiae*, van Niel and Anderson found that as much as 30 per cent. of added glucose was initially converted into complex carbohydrates, while the production of carbon dioxide and ethyl alcohol accounted for the remaining 70 per cent. This was interpreted as further evidence of the occurrence of fermentative assimilation.

However, this could not be demonstrated in lactic acid fermentation. Concerning the nature of the carbohydrates synthesised by yeast, it has been definitely established that apart from glycogen, other compounds such as yeast gum and membrane polyose are also formed. According to the work of McAnally and Smedley-Maclean the addition of phosphate to glucose or maltose media increased the storage of glycogen, yeast gum and insoluble carbohydrate by yeast cells. The amount of glycogen formed from maltose far exceeded that derived from glucose.

The foregoing literature, therefore, affords strong evidence that carbohydrate metabolism as studied in a variety of micro-organisms including yeast strains, is closely associated with the phenomena of oxidative or fermentative assimilation. By these processes variable amounts of added carbohydrate are either assimilated by the organisms and synthesised into reserve carbohydrate (glycogen) or rapidly oxidised with the production of gas (CO_2) and water. The relative extent to which either assimilation or oxidation proceeds, as well as the speed of reaction in these two phases of bacterial metabolism, depends on a variety of factors in which the type of organisms, the amount and nature of their food supply, and the degree of aeration appear to be of the greatest significance. The present investigations of the yeast strain *Schizosaccharomyces ovis* as well as other species of iodophilic micro-organisms, have revealed the fact that fundamentally similar processes of oxidative assimilation are of normal occurrence within the forestomachs of sheep and probably also in other species of ruminants.

Conclusion.

In studies conducted on merino sheep with permanent ruminal fistulae, it has been demonstrated that acute gas production in the forestomachs immediately after the consumption of certain foods is associated with a process of oxidative assimilation. By this process variable proportions of such sugars as glucose, fructose, and sucrose are rapidly oxidised through the agency of a strain of false yeast, *Schizosaccharomyces ovis*, which is present in large numbers in the rumen of sheep, especially when such animals are kept on a diet of lucerne. Attending this oxidation of part of the ingested sugar, large volumes of gas are suddenly generated within the ruminal mass. Simultaneously with this, the rest of the sugar is rapidly assimilated and stored as glycogen by the yeast cells. Complete starvation or inadequate feeding of the animal is promptly followed by suppression leading up to a total disappearance of this yeast strain. Under these circumstances various iodophilic bacteria normally present in the ruminal ingesta are afforded the opportunity of metabolising the available sugar. This is associated with the synthesis of starch by these organisms instead of glycogen. Moreover, oxidation shows greater restriction as is evident from the reduced amount of gas produced.

While this extensive synthesis of glycogen and other polysaccharides forms an integral part in the carbohydrate metabolism of various ruminal micro-organisms, its full significance in the biology of the microflora and especially in the nutrition of the host animal itself is as yet not fully understood. In view of the close relationship existing between ruminant digestion and bacterial activity there are indications, however, that the nutrition of ruminant animals is vitally linked with various products derived from bacterial metabolism, hence the necessity of further investigations in this field.

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