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The Utilization of Non-protein Nitrogen in the Bovine Rumen

7. A QUALITATIVE AND QUANTITATIVE STUDY OF THE BREAKDOWN OF CARBOHYDRATE WHICH ACCOMPANIES PROTEIN FORMATION IN BOVINE RUMEN CONTENTS DURING *IN VITRO* INCUBATION

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In an earlier paper of the present series Pearson & Smith (1943*a*) showed that when fresh rumen contents were incubated *in vitro* both synthesis and hydrolysis of protein could readily occur, but that to obtain the prolific bacterial growth which results in protein synthesis predominating over protein hydrolysis, an ample supply of readily available carbohydrate was essential. It would appear probable, therefore, that the non-protein nitrogenous constituents of feeding stuffs may be of value in the rumen only when there is also an abundance of simple sugar or starch in the diet, and since the products commonly fed to ruminants contain many different types of carbohydrates and related compounds, it seemed important to determine to what extent protein synthesis predominated in rumen contents when a variety of different substances were made available as possible sources of energy for the bacteria. It was hoped also that the work would give information regarding the types of compound on which rumen micro-organisms can act. Pearson & Smith (1943*a*) had already shown that when soluble starch, D-(+)-galactose, sucrose, maltose, lactose and D-(+)-glucose were added to rumen liquid during incubation *in vitro*, vigorous bacterial proliferation resulting in the predomination of protein synthesis occurred. The present communication describes the extension of that work to include other naturally occurring carbohydrates, such as arabinose and xylose, and also certain derivatives of carbohydrates. Several other substances including lower fatty acids, lactic acid and succinic acid have also been tested.

It is now well established that much of the carbohydrate in the diet of ruminating animals is fermented in the rumen to form various end products which include lower fatty acids, carbon dioxide and methane (Phillipson, 1947-8; Elsdon & Phillipson, 1948), but since the rumen is part of an open system from which these products can pass further along the alimentary tract or can be absorbed into the blood or be excreted in the breath, it is not easy to estimate *in vivo* the proportions in which these various substances are produced. It seemed possible, however, that by adapting the *in vitro* incubation technique of Pearson & Smith (1943*a*) so that the gaseous products of fermentation could be collected and analysed, it would be possible to determine approximately, but with more accuracy than hitherto, the relative proportions in which the various end products were formed from different sugars, and how much of the utilized sugar could be accounted for by the end products. Since maltose and the pentoses arabinose and xylose are all known to occur as units of more complex carbohydrate molecules in vegetable products, these three carbohydrates were used in this quantitative section of the work.

The objects of the present investigation were first to find what types of compound could be utilized by the rumen bacteria to enable protein synthesis to predominate over protein hydrolysis, and secondly to determine the proportions in which the main end products of carbohydrate fermentation in rumen contents are formed, how much of the fermented carbohydrate can be accounted for by these pro-

ducts, and whether the end products with a disaccharide like maltose, which consists of hexose units, differ from those formed when the fermented sugar consists mainly of a pentose such as arabinose or xylose.

METHODS

The method of obtaining and incubating samples of fresh rumen contents from animals with a permanent rumen fistula has been described by Pearson & Smith (1943*a*). Since, however, Smith & Baker (1944) showed that the rumen protozoa appeared to play no part in the marked protein synthesis which they obtained in their brief *in vitro* incubations, the protozoa were removed from the rumen liquid by light centrifugation (5 min. at 2000 rev./min., approximately 800 g) before incubation began. Urea (0.05%) was added to all samples of rumen liquid to ensure that lack of non-protein nitrogen (N.P.N.) did not in any way limit bacterial activity. Unless otherwise stated, carbohydrates and other test substances were added at a level of 1%. The period of incubation in all experiments was 4 hr. For experiments in which CO₂ and CH₄ production were measured, the incubation flasks were fitted with air-tight bungs carrying inlet and outlet tubes, and a slow stream of CO₂-free air was drawn through the incubating material to remove the gaseous products to the aspirators in which they were collected. Further details are given later (p. 328).

The amount of protein synthesis occurring during incubation was taken as a measure of bacterial activity. Samples from the incubation flask were therefore preserved before and after incubation for chemical analyses.

Methods of analysis

Nitrogen. The N.P.N. and protein N of the rumen liquid were partitioned by the sodium tungstate procedure of Pearson & Smith (1943*c*).

Carbohydrates. The sample of rumen liquid was first separated into bacterial sediment and supernatant liquid by centrifugation as described by Smith & Baker (1944). The reducing power of these fractions before and after acid hydrolysis was then determined in order to obtain the amount of bacterial polysaccharide and soluble carbohydrate which each contained. For this purpose the gravimetric Cu₂O method of Munsen & Walker, as recommended by the Association of Official Agricultural Chemists (1945), was used. Where pentoses were being studied, the amount present in the bacterial sediment and supernatant liquid was measured by the furfural-phloroglucide procedure, as described by the Association of Official Agricultural Chemists (1945).

Total volatile fatty acids. These were estimated by exhaustive steam distillation after acidification with 10N-H₂SO₄. In a typical experiment 50 ml. of rumen liquid were acidified with 10 ml. of 10N-H₂SO₄ and about 1 l. of distillate collected. The distillate was titrated with 0.1N-NaOH and the acidity expressed in terms of acetic acid.

Volatile fatty acid partition. The acids were first separated from the rumen liquid by the double distillation procedure of Friedemann (1938). The silica-gel partition chromatographic technique of Moyle, Baldwin & Scarisbrick (1948) was used to separate the acids into acetic, propionic and butyric acid fractions. The last fraction contained a very small amount of a higher acid which was not identified.

Lactic acid. The method of Barker & Summerson (1941) was found to be satisfactory and was adopted.

Gas analysis. When samples of gas were collected from the rumen incubations they were analysed for CO₂ and CH₄ in a Haldane apparatus.

RESULTS

The bacterial activity in rumen liquid in the presence of carbohydrates, derivatives of carbohydrates, lower fatty acids and other compounds

In this section of the work a sample of rumen liquid, prepared as already described, was divided into the required number of portions. To one portion, which served as a control, 1% maltose was added; to the others 1% of a test substance was added. In some experiments one portion of the rumen liquid was incubated without addition of carbohydrate or other source of energy to serve as a second type of control. As shown in Table 1 the activity resulting from each incubation was expressed in terms of the amount of N.P.N. converted to protein N.

Carbohydrates. The tests made with maize and potato starch (Table 1) showed that when these starches were added to the rumen liquid in the raw state, bacterial activity was much less than with maltose. However, where the solutions of the starches were boiled before use, each produced an amount of bacterial growth considerably greater than that obtained with maltose.

Additions of raffinose, inulin, D-(−)-fructose, and cellobiose each resulted in an amount of protein synthesis of the same order as that brought about by the addition of maltose. This suggests that the rumen bacteria were able to metabolize not only the sugars, fructose, glucose and galactose, but also to break down the types of linkage which exist between the monosaccharide units in raffinose, inulin and cellobiose. The simple sugar, D-(+)-mannose was also as well utilized as maltose, but the bacteria appeared to be unable to use L-(−)-sorbitol. This lack of protein synthesis in the presence of sorbitol might have been due to its configuration or possibly to a trace of some toxic impurity in the sugar. Sorbitol from two different sources was therefore tested. One was a sample obtained commercially, and even after recrystallization it did not result in protein synthesis. The other sample was kindly supplied by Prof. E. L. Hirst, F.R.S., but it also caused no measurable amount of bacterial activity. It would appear probable, therefore, that it was the configuration of the sorbitol molecule which rendered it useless to the bacteria.

Of the three pentoses, L-(+)-arabinose and D-(+)-xylose appeared to be well utilized (Table 1), but the remaining one, D-(−)-arabinose, did not result in nearly so much activity as the others. It is interesting to note in this connexion that L-(+)-arabinose is the form of that pentose which occurs most

Table 1. *Protein synthesis in rumen liquid when different substances were added as possible sources of energy for the bacteria*

(Unless otherwise stated, 1% of each substance was added.)

Type of substance added	Control		Experimental	
	Substance added	Protein synthesis (+) or hydrolysis (-) (mg. N/100 g. rumen liquid)	Substance added	Protein synthesis (+) or hydrolysis (-) (mg. N/100 g. rumen liquid)
Carbohydrate	Maltose	+8.9	{ Raw potato starch	+2.9
			{ Boiled potato starch	+15.5
			{ Raw maize starch	+5.5
			{ Boiled maize starch	+15.8
	Maltose	+9.5	{ Raffinose	+7.4
			{ Inulin	+9.2
	Maltose	+13.0	{ Fructose	+14.0
			{ Cellobiose	+13.5
	Maltose	+5.7	{ Mannose	+5.5
			{ Sorbose	-3.0
Compounds related to hexoses	{ Maltose	+17.6	{ L-(+)-Arabinose	+21.4
	{ No addition	-2.0	{ D-(-)-Arabinose	+3.1
	Maltose	+7.0	{ D-(+)-Xylose	+18.7
			{ Glucuronic acid	-0.6
	Glucose	+7.1	{ Gluconic acid	-1.0
			{ Glucosaccharic acid	-0.2
	Maltose	+5.7	{ Mannitol	+0.6
			{ Sorbitol	+0.6
	Maltose	+3.6	{ Alginic acid	+0.9
			{ Glucosamine	+0.7
Lower fatty acids	{ Maltose	+7.2	{ Sodium acetate	+0.4
	{ No addition	-1.6	{ Sodium butyrate	-1.3
	{ Maltose	+9.6	Sodium propionate	-1.7
	{ No addition	-3.7		
	{ Maltose	+9.6	Sodium β -hydroxybutyrate	+3.5
	{ No addition	+1.6		
Other organic acids	Maltose	+9.2	{ Lactic acid (0.5%)	-0.5
			{ Sodium citrate	-2.7
	Maltose	+14.1	{ Sodium hydrogen malate	-1.2
			{ Sodium potassium tartrate	-1.3
	No addition	-2.2	{ Fumaric acid	-0.5
			{ Succinic acid (0.3%)	-2.4
	Maltose (0.3%)	+2.3		

commonly in natural products. Since both arabinose and xylose residues may form quite a considerable proportion of the carbohydrate present in vegetable products it is important to know that these pentoses can be utilized so readily by rumen bacteria.

Compounds related to hexoses. The results in Table 1 show that the three oxidation products of glucose, namely glucuronic, gluconic and glucosaccharic acids, when compared with both maltose and glucose, did not enable protein synthesis to predominate. The same was true of the reduction products of fructose and glucose, namely, mannitol and sorbitol, and also of alginic acid and glucosamine.

Lower fatty acids. The results of tests made with these acids are also shown in Table 1. The addition

of sodium acetate, propionate or butyrate caused no apparent activity. It would appear, therefore, that these lower fatty acids, which are known to be formed during fermentation in the rumen, are end products and are not readily utilized further by the bacteria.

Other organic acids. The acids tested were β -hydroxybutyric, lactic, citric, malic, tartaric, fumaric and succinic. With the possible exception of β -hydroxybutyric acid none of them appeared to cause any marked bacterial activity. In the test with lactic acid it was found that initially 585 mg. lactic acid per 100 g. rumen liquid were present and that after 4 hr. the value appeared to have decreased to 561 mg., a change which can scarcely be regarded as significant.

A quantitative study of the products formed from maltose, xylose and arabinose by rumen bacteria during in vitro incubation

The object of this section of the work was to determine the proportions in which the main end products of carbohydrate fermentation in rumen contents are formed and to find how much of the fermented carbohydrate can be accounted for by the formation of these products.

Incubation technique with gas collection procedure. In a typical experiment 2 l. of rumen liquid to which 1% of sugar and 0.05% of urea had been added were divided into two equal portions. One portion, which was contained in a flask fitted with a bung carrying air inlet and outlet tubes, was immediately placed in the incubation bath. The flask was connected to a 10 l. aspirator containing 14 N-H₂SO₄. By slowly emptying this aspirator a CO₂-free air stream was drawn slowly and continuously through the flask to sweep out and collect the gases produced during incubation.

Samples of the other portion were preserved by the addition of H₂SO₄ for the determination of lactic acid and lower fatty acids and by the addition of HCl for the determination of soluble sugars and polysaccharide. A sample preserved with 1% formalin was also kept for N determinations. To obtain a measure of the total CO₂ including CO₂ of carbonates and bicarbonates present initially in the rumen liquid, a 200 ml. sample of this portion of the unincubated rumen liquid was placed in a flask and connected to gas collection apparatus similar to that described for the incubation flask. After acidification with H₂SO₄ a brisk current of CO₂-free air was drawn through the liquid and approximately 2 l. of the gas mixture collected over 14 N-H₂SO₄. This procedure also gave an estimation of the amount of CH₄ initially present in solution in the rumen liquid.

At the end of the 4 hr. incubation period samples of the incubated rumen liquid were again preserved for analysis. CO₂ and CH₄ remaining in solution were removed as before by acidification followed by brisk aeration, and estimated.

Substances produced during the in vitro incubation of rumen liquid in the presence of added carbohydrate

Gaseous products. In each experiment the three gas samples, collected as described, were analysed for carbon dioxide and combustible gas in a Haldane gas analysis apparatus. As a result of combustion the volume of carbon dioxide produced was found to be twice the volume of oxygen used, which suggests in experiments of this type that the combustible gas was mainly, if not entirely, methane. Since, however, the presence of small proportions of other combustible gases such as olefines and carbon monoxide have been reported in gases from the rumen (Olsen, 1942; Dougherty, 1941) samples of gas collected during a typical experiment were treated in an Orsat-Fischer gas analysis apparatus with bromine water to test for olefines and with cuprous chloride to test for carbon monoxide. Olefines and carbon monoxide could not be detected.

In calculating the amount of carbon dioxide which had been produced from the carbohydrate, allowance was made for the carbon dioxide which would theoretically be liberated from added urea by urease activity (Pearson & Smith, 1943b) and also from carbon dioxide liberated from carbonates and bicarbonates as a result of acid production during incubation. Methane figures were also corrected for any methane originally present in solution at the beginning of incubation and removed by aeration during the incubation period. The amounts of both gases formed in experiments with maltose, xylose and arabinose are recorded in Table 2. In presenting the results the fermentation products have been expressed in terms of the carbon they contained so that the amounts formed can be compared on a common basis with the amount of carbohydrate fermented.

Table 2. *Amount of carbohydrate expressed in terms of carbon which was utilized during incubation and which was accounted for by the carbon of the various end products formed*

(Three experiments were made with maltose, one with arabinose and two with xylose.)

Added carbohydrate ...				Amount formed during incubation (mg. C/100 g. rumen liquid)						Percentage of carbohydrate C accounted for by each product					
				Maltose			Arab- inose	Xylose		Maltose			Arab- inose	Xylose	
Exp.	...	...	...	1	2	3		1	2	1	2	3		1	2
End products															
Volatile fatty acids				88*	44*	104*	62*	77	59	35.8	23.6	44.8	41.3	52.4	44.5
Lactic acid				14	18	20	1	0	0	5.7	9.6	8.6	0.7	0.0	0.0
Carbon dioxide				18	13	22	6	12	2	7.3	7.0	9.5	4.0	8.2	1.5
Methane				8	3	10	2	4	2	3.3	1.6	4.3	1.3	2.7	1.5
Bacterial protein				16	28	32	25	21	24	6.5	15.0	13.8	16.7	14.3	18.0
Bacterial polysaccharide				76	72	34	28	22	24	30.9	38.5	14.7	18.7	15.0	18.0
Total				220	178	222	124	136	111	89.5	95.3	95.7	82.7	92.6	83.5
Carbon of carbohydrate changed during incubation				246	187	232	150	147	133						

* These figures were obtained by estimating total acidity and applying the factor derived in Table 3.

Volatile fatty acids. The results for fatty acids given for four of the experiments in Table 2 are marked with an asterisk to denote that only total volatile acidity was determined and that the figures shown were calculated by using a factor obtained from later experiments in which the acids were partitioned into acetic, propionic and butyric fractions. The results of these later experiments are shown in Table 3, which gives details of fatty acid production in tests in which the silica-gel partition

Bacterial protein. In calculating the amount of carbon in bacterial protein, the assumptions were made that all the nitrogen of the sediment isolated was protein and that the nitrogen content of bacterial protein is 16% and the carbon content 53%. Table 2 shows that from 6.5 to 18% of the carbon of the fermented sugar was recovered as bacterial protein.

Bacterial polysaccharide. Since the bacterial sediment gave a strongly positive reaction when tested

Table 3. *Composition of fatty acid mixtures produced during in vitro incubation of rumen liquid with maltose, arabinose and xylose*

Diet of animal from which rumen liquid was obtained	Exp. no.	Carbohydrate added	Fatty acid production during incubation (ml. 0.1 N-acid/100 g. rumen liquid)				Carbon content of acids produced		Ratio, Acetic/ propionic
			Acetic	Propionic	Butyric	Total	(mg. C/ 100 g. rumen liquid)	(mg. C/ ml. 0.1 N- acid)	
Pasture grass	1	Maltose	10.5	10.5	—	21.0	63.0	3.00	1.00
	2	{ Maltose	11.6	12.0	1.8	25.4	79.7	3.14	0.97
		{ Arabinose	12.6	14.2	1.5	28.3	88.6	3.13	0.89
Indoor diet of concentrates and hay	3	{ Maltose	9.3	5.0	4.2	18.5	60.5	3.27	1.86
		{ Xylose	12.3	9.2	3.1	24.6	77.5	3.15	1.34
	4	{ Maltose	10.0	6.7	0.7	17.4	51.5	2.96	1.49
		{ Xylose	12.3	8.6	—	20.9	60.5	2.90	1.43
							Mean	3.08	

chromatogram technique was employed. Some of the tests were made with rumen contents obtained from animals at pasture, and some with rumen contents from animals which were fed indoors on hay, oats, dried grass and beans. The results show that with a grass diet the rumen contents tended to produce acetic and propionic acid in equimolecular proportions, whereas with rumen contents obtained when the animals were stall-fed, the molecular ratio of acetic acid to propionic acid was considerably greater than 1. Butyric acid was produced with some samples of rumen contents, but with others no measurable amount could be detected. This difference between samples was not related to the type of diet fed to the animals nor did it depend on whether the sugar being utilized was maltose, arabinose or xylose. For the present work, the most important fact arising from Table 3 is that the carbon content of the acids produced (expressed as mg. C/ml. 0.1 N-acid) varied only within relatively narrow limits and averaged 3.08. Where, therefore, a factor was used to obtain the figures in Table 2, this value was adopted.

Lactic acid. Lactic acid production figures are given in Table 2. It is in these figures that the most striking difference existed between the results with maltose and those for the two pentoses. From 5 to 10% of the fermented maltose was recovered as lactic acid, whereas less than 1% of the arabinose and none of the xylose could be accounted for by formation of this acid.

for starch with iodine the bacterial polysaccharide was calculated as starch. In two of the maltose experiments approximately one-third of the fermented maltose carbon was recovered as polysaccharide, whereas in the other four experiments only about one-sixth was recovered in that form. In an early experiment with pentose, the staining reaction did not indicate the presence of starch, suggesting that possibly a pentosan was being stored. In the present experiments, however, tests for pentose in the bacterial sediment showed that sediments from hexose and pentose incubations contained equal amounts of pentose (4.1% in one experiment in which maltose and arabinose were compared), indicating that there had been no greater deposition of pentosan by the organisms which were utilizing pentose.

The changes undergone by various rumen liquid constituents during in vitro incubation in the absence of added carbohydrate

In calculating the recovery of added carbohydrate as volatile fatty acid, etc., in Table 2 the assumption was made that the various fermentation products estimated arose from the added carbohydrate. The possibility that some of these might be formed in significant amounts from other constituents of the rumen liquid even during an incubation period of 4 hr. had also to be considered. To investigate this point two separate experiments were carried out in which rumen liquid was incubated under conditions

similar to those already described except that no carbohydrate was added. Under these circumstances very little change could be detected. Protein was the main constituent to be affected, but the amount decomposed in 4 hr. was equivalent to only about 9 mg. carbon/100 g. rumen liquid, whereas when 1% sugar was present, the change which occurred in the same time was equivalent to as much as 133–246 mg. carbon/100 ml. (Table 2).

DISCUSSION

The first section of the work has confirmed that if in 4 hr. incubations the vigorous bacterial growth observed by Pearson & Smith (1943*a*) and by Smith & Baker (1944) is to occur, the presence of dissolved starch or sugar is essential. From the present work and that of Pearson & Smith (1943*a*) it is clear that in addition to starch, the bacteria are able to use the polysaccharide inulin, the trisaccharide raffinose, the disaccharides maltose, lactose, cellobiose and sucrose, the hexoses D-(+)-galactose, D-(+)-mannose and D-(−)-fructose, and the pentoses L-(+)-arabinose and D-(+)-xylose. They were unable to use L-(−)-sorbose and showed only limited activity with D-(−)-arabinose. Apparently, therefore, they can readily utilize many different carbohydrates, but it is clear that with some sugars the configuration of the molecule may inhibit the ability of the bacteria to ferment them. The necessity for the presence of a potential reducing group and a primary alcohol group in the molecule if it is to be utilized by the bacteria was made clear by the fact that oxidation products of glucose such as gluconic, glucuronic and glucosaccharic acids, and a reduction product such as mannitol did not result in marked protein synthesis.

In addition to sugars and related compounds, volatile fatty acids were tested. It is well established that they are products of fermentation in the rumen and the tests were made to determine whether they were further decomposed by rumen bacteria in the absence of added carbohydrate. The results indicated that in 4 hr. incubations they were not utilized. These observations agree with the conclusions of Phillipson & McAnally (1942), who found that the volatile fatty acids were stable in rumen contents for at least 7·5 hr. In the same experiments Phillipson & McAnally found that lactic acid disappeared rapidly from the rumen, but that when rumen contents were filtered through muslin, they had a variable effect upon lactate *in vitro*, and that the supernatant liquid obtained by centrifuging rumen contents had no effect. Elsdon (1945–6) found that during prolonged *in vitro* incubation of diluted rumen contents with glucose, lactic acid accumulated during the first stage of fermentation and was later changed to volatile fatty acid. In the present

work lactic acid did not appear to be changed to any significant extent when incubated with fresh rumen contents for 4 hr. Several other acids such as citric, tartaric and succinic were tested, but they did not induce any vigorous bacterial growth as measured by protein synthesis.

The second part of the work was designed to study the quantitative aspect of carbohydrate breakdown in rumen contents. It was found that in three experiments with maltose 90, 95 and 96 % of the carbon of the fermented sugar could be accounted for by the formation of acetic, propionic, butyric and lactic acids, carbon dioxide, methane and bacterial polysaccharide and protein. With xylose the corresponding figures were 84 and 93 %, and in the one experiment with arabinose the value was 83 %. The fact that 4–10 % was unaccounted for with maltose, and as much as 17 % with the pentoses, may be attributed to two causes. In the first place the determinations of the several end products are subject to errors, some of which may well be cumulative. For example, Heald (1951) has shown that not all of the reducing substances obtained on hydrolysing rumen micro-organisms are fermentable sugars. Consequently the use of reduction methods for estimating sugars in complex work of this type cannot be other than approximate. Heald found, however, that the amount of non-fermentable reducing substances in the rumen contents of sheep remained fairly constant throughout the digestive cycle. It would seem, therefore, that by measuring changes in the total reducing substances a fair estimate of the changes in fermentable material would be obtained.

In the second place, only the main products of the bacterial activity were determined. Marston (1948), who described somewhat similar experiments on the fermentation of cellulose in rumen contents, in which he obtained recoveries of about 96 %, tested for intermediate products such as succinic acid, acetaldehyde, other aldehydes and ketones, lactic acid and pyruvic acid, and concluded that they did not appear in quantities sufficient to affect the recovery figures. Of these substances only lactic acid was tested for in the present work. It was formed in significant amounts from maltose, but not from the two pentoses. Since the recoveries with the pentoses tended to be less (83–93 %) than those obtained with maltose (90–96 %), it may well be that when pentoses are decomposed in rumen contents some compound, not estimated in the present studies, is formed instead of the lactic acid which is formed from maltose. This point requires further investigation.

In Marston's experiments cellulose was used as the source of carbohydrate and incubations were necessarily much longer than in the present work. The technique also differed in that the micro-organisms were first isolated from rumen contents,

washed and suspended in an inorganic medium containing cellulose. Marston's results differed from the present findings in several aspects. For example, the average amount of cellulose carbon recovered as lower fatty acids was 73 % and as gaseous products 21 %, very little being accounted for by the formation of bacterial protein and polysaccharide. In the 4 hr. sugar incubations of the present work, a very much higher proportion of the recovered carbon was present in the bacteria. This observation agrees with the theory that maximal bacterial proliferation and utilization of N.P.N. occurs during the short period of vigorous fermentation which follows a feed, when readily available carbohydrate is rapidly broken down. In this connexion it has been shown in feeding experiments by Rupel, Bohstedt & Hart (1943) and by Willett, Henke & Maruyama (1946) that maximum N.P.N. utilization occurs when the diet is rich in carbohydrates which the bacteria can utilize readily and rapidly.

Very little information is available in the literature regarding the amounts of the various products which are formed from simple sugars in the rumen of intact animals, but Phillipson & McAnally (1942) observed that when 100 g. galactose, a sugar which they found behaved similarly to maltose in the rumen, was given to a sheep, the increase in volatile fatty acids calculated as acetic acid in the rumen liquid amounted to 170 mg./100 ml. within 4.5 hr. The carbon content of this amount of acetic acid is 68 mg. and is within the range of volatile fatty acid production (44–88 mg. C/100 g. rumen liquid) found in the present investigations (Table 2).

The figures for the production of carbon dioxide and methane in the present work varied considerably from one experiment to another, and the ratio of carbon dioxide to methane formed varied from 1.0 to 4.3. The average of these ratios, however, was 2.63, a figure which agrees well with that of 2.58 reported by Kleiber, Cole & Mead (1943) for the rumen gases of intact animals. The present figure is similar also to that of 2.40 obtained by Washburn & Brody (1937) for the gas from the rumen of a cow slaughtered 4 hr. after a feed.

These considerations lead to the conclusion that in the *in vitro* experiments of Pearson & Smith (1943*a, b, c*) and Smith & Baker (1944) the changes which occurred when rumen liquid was incubated with soluble carbohydrate were very similar to those occurring in the rumen itself, particularly in the few hours following a meal. It seems probable, therefore, that conclusions regarding protein synthesis and breakdown drawn from results of the *in vitro* work

described in earlier parts of the present series may reasonably be considered to apply also to the intact animal.

SUMMARY

1. A study has been made of the ability of several carbohydrates and other compounds to support vigorous bacterial growth in bovine rumen contents. Each substance was incubated in fresh rumen liquid at a concentration of 1 %. Portions of the same rumen liquid were incubated with 1 % maltose or glucose as control.

2. The polysaccharides starch and inulin, the trisaccharide raffinose and several disaccharides, hexoses and pentoses were found to be readily utilized by the bacteria. Since oxidation products of glucose such as gluconic and glucuronic acids and a reduction product such as mannitol produced no measurable activity, it would appear that both a potential aldehyde and a primary alcohol group are essential. Molecular configuration was also important since fructose was readily fermented while sorbose showed no activity. Similarly, L-(+)-arabinose gave marked bacterial growth, whereas with D-(-)-arabinose activity was much more limited.

3. The other substances tested included lower fatty acids, lactic acid, succinic and tartaric acids. None of them gave rise to bacterial growth as measured by protein synthesis.

4. A quantitative study has been made of the principal products formed when maltose, arabinose and xylose are utilized *in vitro* by the microflora of the bovine rumen during an incubation period of 4 hr.

5. In three experiments with maltose 90–96 % of the carbon of the maltose fermented was accounted for by the formation of lower fatty acids, lactic acid, carbon dioxide, methane, bacterial protein and polysaccharide.

6. In two experiments with xylose and one with arabinose 83–93 % of the carbon of the decomposed sugar was accounted for.

7. The results for the pentoses differed from those obtained with maltose in that with the pentoses no lactic acid was detected among the end products.

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Ranunculin: The Precursor of the Vesicant Substance of the Buttercup

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Many of the plants belonging to the natural order Ranunculaceae have the property of producing erythema and blistering the skin. From these plants a terpene-like substance can be obtained, which has long been known under the name of anemonin (I). Anemonin, however, has no vesicant properties, and is derived from the active substance by polymerization. The constitution of the active substance known as protoanemonin (II) has been elucidated by Yasuhiko & Fujita (1922) and Kipping (1935). This was shown to be the lactone of γ -hydroxyvinylacrylic acid (III) and is an oil, volatile in steam and very soluble in water. The pure substance or its solution in water polymerizes in the course of a few days to the insoluble crystalline product anemonin. The distribution of protoanemonin among a series of *Ranunculus* spp. was examined quantitatively by Shearer (1938).

The present investigation was undertaken with the object of finding how the protoanemonin was both stable and harmless within the plant itself. A further object was to obtain a readily available supply of protoanemonin for examination of its action on enzymes, so that the properties of this very simple natural vesicant could be compared with those of the well known vesicants.

It has been found that when the tissues are crushed the protoanemonin is liberated enzymically from a

glucosidic precursor in the plant. The glucoside itself has been isolated as a crystalline substance very soluble in water, and, unlike protoanemonin itself, is stable both as a solid and in aqueous solution. This substance, for which structure (IV) is suggested, we propose to call ranunculin, as it has been obtained from several species of *Ranunculus*. Ranunculin, while very stable in acid solution, is rapidly broken down in alkaline solution with the liberation of glucose. The protoanemonin thus formed is converted into acetylacrylic acid (V). Ranunculin, on distillation with aqueous sodium acetate, gives a nearly quantitative yield of protoanemonin and is thus a convenient stable source of the latter.

The ease of production of protoanemonin (II) suggests that in the glucoside the sugar residue is attached to a substance closely related to the lactone. Protoanemonin itself could not be regarded, however, as the aglucone because it has no free hydroxyl group. The most natural assumption would be that the glucoside is that of the enol form of acetylacrylic acid. However, as neither the glycoside nor the tetra-acetyl derivative shows the presence of a free carboxyl group, this assumption could not be true. The rapid liberation of the glucose by alkali and the relative stability of the glucoside in acid solution would point to its being a glucose ester of acetylacrylic acid. This, however, will not easily explain the ready formation of the lactone by boiling with sodium acetate solution or as the initial product of

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