STUDIES IN RUMINANT METABOLISM

A Thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Science.

by

MARY LINDSAY McNAUGHT

May, 1948

The Hannah Dairy Research Institute
Kirkhill, Ayr

CONTENTS

SYNOPSIS

Part I. Experiments on the Value of *in vitro* Studies, and on the Decomposition of Carbohydrate by Rumen Liquid

Most of the work described in the present thesis involved incubating rumen contents in the laboratory under conditions which were designed to resemble those existing in the normal rumen. Before adopting this technique, however, it was important to determine whether the changes which took place in the incubating material *in vitro* were similar to those which are known to occur *in vivo*. To obtain information on this matter the decomposition of carbohydrate during *in vitro* incubation was studied. It was shown that the amounts of volatile fatty acids, lactic acid, carbon dioxide and methane produced from maltose in the laboratory were comparable to the amounts produced *in vivo*. It may be concluded, therefore, that the nitrogen changes which invariably accompany these carbohydrate changes *in vitro* also occur *in vivo*.

Of the maltose decomposed during incubation, 81-90% was accounted for by the production of bacterial protein, bacterial polysaccharide, carbon dioxide, methane, lactic acid and volatile fatty acids. The remaining 10-19% may be accounted for partly by

the fact that volatile fatty acids were calculated as acetic acid whereas almost certainly propionic and butyric acids also occurred, and partly by the production in small amounts of substances not so far identified.

Part II. The Preparation of a dried Sample of Rumen Bacteria and the Biological Value of its Protein

The results of many feeding trials and *in vitro* studies described in the literature have suggested that non-protein nitrogen (N.P.N.) may be converted to microbial protein in the rumen and that this protein becomes available to the ruminating animal. To determine the biological value of this microbial protein it was decided to prepare a sample of dried rumen bacteria sufficient in amount to enable feeding tests to be made with rats. Approximately 460 g. of dried rumen bacteria were prepared and analysed. The preparation contained 44% protein, of which about two-fifths was synthesised from N.P.N. during incubation. Microscopical and chemical examination of the material suggested that contamination by fibre and protein from feedingstuffs was very slight.

When fed to rats at a level of 8% of the diet the biological value of the protein was 88.2% and the digestibility 73.2%.

Work similar to that described for rumen

bacteria has been begun with rumen protozoa.

Part III. The Effect of Animal Management Factors on the Synthesising Power of Rumen Liquid in vitro

The synthesising power of rumen liquid as judged by the decrease in N.P.N., which occurs on incubating the liquid with added carbohydrate under controlled conditions in the laboratory varies considerably from time to time. Attempts have been made to relate these variations to differences in protein or N.P.N. content of the rumen liquid when the animal was fed different diets. However, apart from the finding that the protein and N.P.N. levels and the amount of synthesis were all higher when the animals were at pasture than when they were stall-fed there appeared to be no definite relationship between the diet, the naturally occurring level of N.P.N. or protein in the rumen liquid and the amount of synthesis. An apparent increase in protein synthesis was detected when urea was added to rumen liquid, but further work is required to determine whether the increase was real. The effect of the time of sampling on synthesis was also investigated, and it was shown conclusively that maximum synthesis occurred in vitro, in samples which had been removed from the rumen approximately one hour after the animal had been fed.

Part IV. The Effect of Aeration and of the Nature of the Carbohydrate present on the Amount of Protein Synthesis occurring in vitro

The effect of some factors which may be controlled in the laboratory, on the amount of synthesis occurring *in vitro* was investigated. It was shown that incubation of rumen liquid in a flask aerated with air, oxygen, nitrogen or carbon dioxide did not result in better synthesis than incubation in a flask loosely stoppered and subjected to occasional gentle agitation. Aeration with air or oxygen actually depressed synthesis.

The effect of adding different substances as sources of energy for the bacteria was also investigated. Of the sugars tested only sorbose, which may have contained some toxic impurity, and the D (-) isomer of arabinose were of no value. Those which could be utilized were starch, inulin, raffinose, maltose, fructose, glucose, mannose, L (+) arabinose and D (+) xylose. Glucose -1-PO_4 was found to be a ready source of energy. Compounds such as alginic acid, glucosamine, gluconic acid, glucuronic acid, saccharic acid, mannitol and sorbitol were not utilized, nor were acetic, butyric, succinic and several other organic acids.

Preliminary experiments with D (+) xylose and L (-) arabinose showed that fermentation of these sugars resulted in production of the same amount of volatile

fatty acids as fermentation of maltose, but that the amount of lactic acid produced was exceedingly small. The amount of fermented pentose unaccounted for by the various end-products studied was 26% as compared with 10-19% for maltose.

Part V. The Effect of certain Metals on the Amount of Protein Synthesis obtained in vitro

Cobalt and copper, or ferric salts containing cobalt or copper may be supplied to ruminants to control deficiency diseases such as swayback, pining and falling disease. Molybdenum occuring in abnormally high concentration in pastures causes digestive disturbances. The effect of various concentrations of cobalt, copper, iron and molybdenum on the amount of protein synthesis occurring in vitro was, therefore, determined. Lead was also tested. The results show that copper has the most powerful inhibitory effect on protein synthesis, but the amount required to produce the inhibition is above that normally administered. Similarly cobalt was only toxic in concentrations far above what would normally be taken by cattle. Molybdenum was less toxic than cobalt or copper. The possibility of iron or lead affecting the rumen organisms under practical conditions is extremely slight.

Part VI. Experiments with "Coated" Urea

In an attempt to find a means of retarding the rate at which urea dissolves in rumen liquid so that it might be liberated slowly into the rumen contents throughout the day instead of immediately after feeding, some samples of urea coated with various proportions of starch and with stearic acid and cellulose acetate were prepared by Imperial Chemical Industries Ltd. The rate at which the rumen liquid extracted urea from these compounds and converted it to ammonia was studied. Similar tests were made with feedingstuff cubes containing urea. It was found that urea was extracted completely from all the coated samples and also from the feedingstuff cubes within 20 minutes, and that the coated samples had no advantage over urea incorporated in feedingstuff cubes.

GENERAL INTRODUCTION

The importance of ruminants as converters of fibrous feedingstuffs to meat, milk and wool is due largely to their ability to digest cellulose more efficiently than other animals. They are able to extract from the cellulosic structures of the feedingstuffs much of the protein and other nutrients which they contain. Much of the research work carried out during the past fifty years on ruminant metabolism has therefore been concerned with the digestion of cellulose in the rumen. As a result of these and other studies the rumen can now be regarded as a large fermentation "vat" in which the cellulose and other carbohydrates are fermented by the prolific microflora normally present. It is also now realized that the microorganisms of the rumen not only break down some of the more complex carbohydrate components of feeding-stuffs but also build up substances of great value to the host animal such as vitamins and proteins.

The whole subject of rumen metabolism was reviewed recently in considerable detail by Baker, Harriss, Phillipson, McNaught, Smith, Kon and Porter (1947-8). Two of these authors dealt with the decomposition of structural cellulose, two with nitrogen metabolism, one with the metabolism of fatty acids and two with vitamin synthesis. Earlier less detailed reviews have been published by Goss (1942-3), Hastings (1944), McAnally and Phillipson (1944) and

Owen (1941; 1947). The mechanical importance of rumination has been dealt with by Schalk and Amadon (1928).

Since the present thesis is concerned primarily with nitrogen metabolism in the rumen and particularly with the utilization of noneprotein nitrogen, it will be necessary to consider here some of the earlier work on this particular aspect of the subject. Once it was realized that a considerable portion of the nitrogen of plants was present in the form of non-protein nitrogenous compounds, such as amino-acids, amides, and ammonium salts, the question naturally arose as to whether these non-protein nitrogenous compounds had any nutritive value. Towards the end of last century many experiments were made by German workers with a view to answering this question. Simple nitrogenous compounds such as ammonium salts, asparagine, glycine, and urea were used to replace part of the protein in rations for various types of animals, and the effect on nitrogen retention, live-weight or milk production noted. With non-ruminating animals no utilization of N.P.N. could be detected, whereas with ruminants, although results were conflicting, some workers claimed that N.P.N. had a protein-sparing effect. To explain this effect Zuntz (1891) and Hagemann (1891) were the first to put forward the hypothesis that amide nitrogen might be assimilated by the rumen microorganisms, the resulting microbial protein becoming available to the host animal

when the organisms were digested further along the alimentary tract.

The early German work on the utilization of "amides" was reviewed by Armsby (1911) and Mitchell and Hamilton (1929) and more recently by Krebs (1937). From his survey of the literature Armsby concluded that under certain conditions amides might be of some nutritive value but under no circumstances could they be utilized to the same extent as protein. Even eighteen years later Mitchell and Hamilton came to the conclusion that the case for N.P.N. utilization required further proof. After a further eight years Krebs came to a similar conclusion and maintained that sufficient evidence had not been produced to show that over a long period a growing ruminant could make satisfactory live-weight increases on a ration which was deficient in protein but which was supplemented with N.P.N. Krebs tended to attribute any favourable effect which feeding N.P.N. appeared to have on milk yield to a "diminution of digestive depression" and not to the formation of protein in the rumen.

By 1937, therefore, the Zuntz-Hagemann protein substitution theory had gained little ground. Benesch, however, reviewing the situation in 1941 indicated that as a result of several feeding trials made in Germany, urea nitrogen had been allotted a value equal to half that of protein nitrogen. At that time it was known that at least four urea preparations for cattle-feeding were being manufactured in Germany. Since then it has

been reported that these preparations were used with a fair amount of success during the earlier years of the recent war. The possibility of using N.P.N. compounds to alleviate protein shortages was examined also in other Continental countries. Steensberg (1947) reported that in Denmark urea-feeding trials with lactating cows were disappointing and consequently the use of urea was abandoned. He did, however, point out that the normal Danish feed ration contains beet in such considerable amounts that it was probable that the animals were receiving as much N.P.N. as they could utilize without addition of urea. Breirem (1947) reported that in Norway, feeding trials had indicated that urea, when added to a low protein ration, could be utilized for milk production, but urea feeding was not used in practice. Brouwer (1947) stated that in Holland, urea and ammonium lactate had been tested as protein substitutes and had been found to possess less than half the value of their nitrogen equivalent of protein for growth and milk production. Consequently the use of these N.P.N. compounds in Holland was only recommended for times of extreme protein shortage such as did not occur even during the war years.

In Britain, Owen, Smith and Wright (1943) conducted metabolism trials with lactating cows, in which bloodmeal and urea were compared as sources of nitrogen for milk production. It was found that the average efficiency of utilization of the urea nitrogen varied greatly for different animals but that it

averaged 75% of that of the bloodmeal nitrogen. Feeding trials were also conducted by Bartlett and Cotton (1938) in which urea was found to have a favourable effect on the growth of heifers when added to a low protein ration. In a later experiment, however, Bartlett and Blaxter (1947) did not find urea to be of value for milk production.

In America several well controlled metabolism trials showed that urea nitrogen was certainly of value for maintenance and growth (Harris and Mitchell, 1941 1, 2; Johnson, Hamilton, Mitchell and Robinson, 1942; Harris, Work and Henke, 1943). The value of urea for growth and milk production was also established by extensive feeding trials not involving metabolism studies, (Hart, Bohstedt, Deobald and Wegner, 1939; Mills, Lardinois, Rupel and Hart 1944; Rupel, Bohstedt and Hart 1943; Archibald, 1943; Willett, Henke and Maruyama, 1946). Efficiency of utilization was not as high as that of protein nitrogen but it was sufficiently high to warrant the marketing of a urea-feed compound in the United States for use in regions where carbohydrate is plentiful and protein scarce.

Although the theory of N.P.N. utilization by ruminants gained much ground as a result of these feeding experiments, it was obvious that further work of a more fundamental nature was required to obtain a clearer understanding of the actual mechanism of utilization, particularly when it is realized that the non-protein nitrogen naturally present in many typical

feedingstuffs forms quite a significant proportion of the total digestible nitrogen. In grass for example it may form 25%, and in grass silage as much as 50% of the digestible nitrogen. Information on three main points was essential. First, it was necessary to determine whether protein synthesis did in fact occur in the rumen and if so, to determine the factors controlling it. Secondly, if protein were found to be synthesised it would be necessary to determine its nutritive value. Thirdly, if it were synthesised the amount of protein becoming available to the ruminant in this way would have to be estimated.

To obtain information on the first of these points, a series of experiments was made at Wisconsin (Wegner, Booth, Bohstedt and Hart, 1940, 1941, 1, 2; Mills, Booth, Bohstedt and Hart, 1942; Mills, Lardinois, Rupel and Hart, 1944). Protein synthesis was first demonstrated by inoculating synthetic media containing urea or ammonium bicarbonate with rumen liquid. Later experiments were made with a fistula heifer which was fed rations with and without added urea. By analysing the rumen contents at various times throughout the day, these authors claimed to show (1) that protein synthesis from urea definitely occurred within 4-6 hours of feeding, (2) that protein synthesis from N.P.N. was depressed when the true protein of the rations being fed was above 18%, (3) that protein synthesis was greatest when urea was added to a grain mixture low in protein and (4) that an adequate supply

of carbohydrate, preferably in the form of starch must be present for protein synthesis to occur.

Experiments on the mechanism of N.P.N. utilization have also been made at the Hannah Institute by Pearson and Smith (1943, 1, 2, 3) and Smith and Baker (1944). Preliminary experiments by Pearson and Smith emphasised the difficulties besetting experiments of the in vivo type made by Hart which have just been cited, and led them to the conclusion that valuable information might be obtained by incubating rumen contents in vitro under conditions as similar as possible to those in the rumen. By this technique they showed that when rumen liquid was incubated with urea and carbohydrate, considerable protein synthesis resulted within 4-6 hours. Collaborative experiments by Smith and Baker (1944) showed that the qualitative nature of the bacterial population did not change within these few hours, and by direct counts made in samples of the rumen liquid from these incubations they showed that protein synthesis was accompanied by marked proliferation of iodophilic bacteria. Furthermore, they showed that protein synthesis and hydrolysis normally occurred simultaneously in rumen liquid. In the absence of sufficient carbohydrate to satisfy energy requirements, or if the protein present was very soluble in water, protein hydrolysis predominated over protein synthesis.

The necessity of knowing the nutritive value of rumen bacterial protein in assessing the importance to

the host animal of protein synthesised from N.P.N. has long been recognised. More than forty years ago Müller (1906) attempted to obtain an estimate of the nutritive value of bacterial protein isolated from rumen contents. He found in one feeding trial that nitrogen retention on a ration containing the bacterial protein was similar to that on rations containing albumin and casein. More recently Usuelli and Fiorini (1938) compared growth of chicks on rations containing bacteria, protozoa and also the dried feedingstuff residues isolated from rumen contents. Growth was greatest in the ration containing protozoa and least in that containing the feedingstuff residues. Johnson, Hamilton, Robinson and Garey (1944) estimated the biological value of the protein of rumen protozoa and bacteria. Only enough of the preparations were available to feed to two or three rats, and the values found for the individual rats differed so widely amongst themselves that obviously a more exact estimate of the nutritive value was required.

The problem of estimating the amount of bacterial protein becoming available to the ruminant is fraught with difficulties. Kohler (1940) from the difference between bacterial counts in rumen and intestinal contents, concluded that only about 23 g. might become available daily. Johnson et al. (1944) on the other hand suggested that with rations containing up to 12% of crude protein all the dietary nitrogen whether protein or N.P.N. is converted to

microbial protein in the rumen. From the rate of protein synthesis *in vitro* found by Pearson and Smith (1943, 3) it can be calculated that during the two periods of vigorous fermentation in the rumen following ingestion of food approximately 150 g. bacterial protein might be synthesised. Thus estimates of the amounts of microbial protein becoming available have varied from the negligible amount of 25 g. per day to amounts sufficient to cover the animal's total protein requirements. Elsden (1945-46) rightly maintains that no reliable estimate will be made until the surgical problem of making a satisfactory fistula between the abomasum and duodenum has been solved.

The work to be described in the present thesis is largely an extension of the work of Pearson and Smith (1943, 1, 2, 3) and of Smith and Baker (1944). Use was made of their technique of incubating rumen contents *in vitro*, to study some of the factors controlling protein synthesis in the rumen. Before proceeding to these investigations, however, it was necessary to obtain some evidence that the vigorous fermentation which occurs when rumen liquid is incubated *in vitro* with maltose, and which is always accompanied by protein synthesis, is characteristic of fermentation in the intact rumen. A study was made, therefore, of the decomposition of maltose during *in vitro* incubation of rumen liquid to determine whether the breakdown products were similar to those arising from decomposition of carbohydrate *in vivo*. At the

same time the opportunity was taken of determining how much of the maltose decomposed in typical incubations could be accounted for by the formation of the various end-products which are known to result from fermentation in the rumen, (Part I). The incubation technique was then used to prepare a relatively large quantity of dried rumen bacteria for the determination of the biological value of the bacterial protein, (Part II). Since the amount of protein synthesis occurring *in vitro* in a given volume of rumen liquid was found to vary considerably from time to time, an attempt was made to determine whether these variations might be attributed to differences in the composition of the rumen liquid caused by changes in the diet of the animal, (Part III). One of the most important factors affecting protein synthesis was the presence of a suitable source of energy. Several substances were, therefore, tested to determine whether the rumen bacteria could utilize them, and this led to a study of the decomposition of pentose in rumen liquid, (Part IV). Since metals such as copper, iron, cobalt and molybdenum may be given to ruminants in the treatment of certain deficiency diseases the effect on the amount of protein synthesis occurring *in vitro* when various concentrations of these metals were added to rumen liquid was investigated. Lead was also tested, (Part V). Finally, one of the factors which may diminish the utilization of N.P.N. in the rumen may be the rapidity with which it dissolves in the rumen

liquid and leaves from the rumen either by absorption or by passing further along the alimentary tract. It was suggested that the rate at which urea dissolved might be retarded by coating it with starch or other substances. A number of solubility tests were, therefore, made with samples of coated urea using rumen liquid as the solute, (Part VI).

Plate 1 : A bullock with a rumen fistula

Plate 2 : A closer view of the fistula

Plate 3 : Removing rumen contents

Plate 4 : The types of solid and liquid ingesta which can be removed

METHODS

Incubation Technique

Material for incubation was obtained from two animals, a steer and a cow, each with a large permanent rumen fistula. The method of closing the fistula has been described in detail by Watts (1948), and provides an efficient means of ensuring that the rumen contents are not subjected to aerobic rather than anaerobic conditions except during the short time necessary to remove samples. Plates 1 and 2 show the steer with the fistula closed, and plate 3, the same animal with the fistula opened and rumen contents being removed. The material was removed by means of an aluminium cup and transferred to a flask which was contained in a bucket of sawdust to minimise heat losses. Plate 4 shows four fractions of rumen contents such as can readily be obtained at any one time from the rumen. The solid fraction is found mainly on the surface of the ingesta and the liquid at the bottom. Semi-solid material such as that shown in the third bottle from the left, was the type taken from the rumen for the present experiments.

"Rumen liquid" for incubation was prepared from the ingesta by straining the sample through muslin to remove the coarser vegetable particles which, if allowed to remain, caused difficulty later in sampling the material for chemical analysis. This liquid was rich in protozoa and bacteria and contained

finely divided vegetable matter. For many of the experiments this strained liquid was used, but for others the liquid was cleared of protozoa and small vegetable particles by centrifuging it for 5 minutes at 2,000 r.p.m. (centrifugal force equivalent to approximately 800 x g.). This procedure inevitably removed a proportion of the larger bacteria, but since they constitute a relatively small proportion of the total bacterial population (Smith and Baker, 1944) their partial removal was not important. Any solid material held on the surface by froth was removed by pouring the centrifuged liquid through a "Clover Leaf" filter pad.

The resulting liquid after addition of carbohydrate and usually also of urea was incubated in the dark in a water-bath thermostatically controlled at 39°C, the body temperature of the cow. The incubation flask was covered with a watch glass, and the contents of the flask were gently agitated at regular intervals throughout the period of incubation which was usually 4 hours.

Analysis of Rumen Liquid

Nitrogen The usual Kjeldahl procedure was followed in estimating nitrogen, the catalyst used being a mixture of copper and potassium sulphates and powdered selenium.

Non-protein nitrogen Two methods of preparing the N.P.N. filtrates were used :-

(a) Sodium tungstate Approximately 25 g. rumen liquid were transferred to a 100 ml. conical flask containing 50 ml. N/6 H_2SO_4, the exact amount added being determined by weight. The contents of the flask were then washed into a 500 ml. volumetric flask with N/6 H_2SO_4, 50 ml. of 10% sodium tungstate solution added, and the volume made up to the mark with the N/6 acid. After vigorous shaking the solution was allowed to stand overnight and then filtered through No.42 Whatman filter paper.

(b) Trichloroacetic acid Rumen liquid (usually about 5 g.) was added to an equal volume of 20% trichloroacetic acid and heated for 5 minutes in a boiling water-bath. In experiments in which both protein and N.P.N. were to be determined, the contents of the flask were diluted with distilled water and filtered directly into a Kjeldahl flask. The protein residue was well washed with water and then transferred in its filter paper to a second Kjeldahl flask. When, however, only the N.P.N. content was required, the mixture of rumen liquid and trichloroacetic acid was heated for 5 minutes in a boiling water-bath and then transferred to a 50 or 100 ml. volumetric flask and made up to the mark with water before it was filtered. Portions of the filtrate were taken for N.P.N. determination.

Ammonia To estimate ammonia in the acid-tungstate filtrate a 100 ml. portion was neutralized to phenol red with N sodium hydroxide, 2 ml. being added in excess. The mixture was then distilled in steam for approximately 15 minutes, the distillate being collected in N/50 sulphuric acid. Sometimes a portion of the trichloroacetic acid filtrate was used in the same way.

Soluble carbohydrate The soluble sugars added to rumen liquid in the carbohydrate decomposition experiments were maltose and the pentoses arabinose and xylose. Before estimating these sugars, the microorganisms with any associated polysaccharide were first removed by prolonged centrifuging as described on p. 24 for the isolation of bacterial sediment. Soluble carbohydrate was then estimated by the following methods :-

(a) Maltose A suitable portion of the centrifuged liquid was diluted to 100 ml. with water, and refluxed with 10 ml. of HCl (sp. gr. 1.125) for approximately ½ hr. to hydrolyse the maltose into dextrose. After almost neutralizing the hydrolysate with NaOH, it was diluted to a convenient volume, usually 250 ml. and filtered. The reducing power of the filtrate was estimated by the method of Munson and Walker as recommended by the Association of Official Agricultural Chemists (1945). In this method the sugar solution is heated under carefully controlled conditions with Soxhlet's modification of Fehling's solution. The cuprous oxide produced is weighed, and tables are provided to convert the weight of cuprous oxide obtained to the amount of dextrose in the solution.

It was found convenient and accurate to use grade 4 sintered glass crucibles (Pyrex) to collect the precipitate of cuprous oxide, but the weight of the empty crucible had to be taken at the end of the estimation after the cuprous oxide had been washed from it with nitric acid, since the weight of the crucible decreased to a small extent when the hot alkaline Fehling's solution was poured through it.

(b) Pentose The furfural phloroglucide method as described by the Association of Official Agricultural Chemists (1945) for the estimation of pentose in wines was used. A suitable portion of the centrifuged liquid was diluted to 100 ml. with water and 43 ml. of concentrated HCl added to start the distillation, which was allowed to proceed at the rate of 30 ml. per 10 minutes. To replace the distillate, 12% HCl was added at 10 minute intervals until 360 ml. of distillate were collected. After addition of phloroglucinol the volume was made up to 400 ml. with 12% HCl, the precipitate allowed to settle overnight and then collected in a weighed sintered glass crucible. For weights of phloroglucide between 0.03 and 0.300 g. the formula (a + 0.0052) x 1.0075 was used to convert phloroglucide to pentose, 'a' being the weight of phloroglucide in grams.

Volatile fatty acids Exhaustive steam distillation was used to separate volatile fatty acids from rumen liquid. In a typical experiment 50 ml. rumen liquid were acidified with 10 ml. 10N H_2SO_4 and the mixture distilled in steam until the distillate was no longer acid. The distillate was filtered to remove traces of higher acids present in solid form. Aliquots were then titrated with N/10 NaOH using phenolphthalein as indicator. For calculation purposes the volatile acids were assumed to consist of acetic acid.

Lactic acid The method used was that of Barker and Summerson (1941). Rumen liquid which had been preserved by addition of 10 ml. of 10N H_2SO_4 per 100 ml. was used for the estimation. A 5 to 10 ml. portion was first neutralized to litmus with NaOH and then diluted to 100 ml. A portion of this neutral solution was transferred to a 25 ml. volumetric flask, 2.5 ml. of 20% copper sulphate solution added, and the volume made up to the mark with water. The contents of the flask were then transferred to a centrifuge tube and mixed well with 2.5 g. $Ca(OH)_2$. After standing for at least $\frac{1}{2}$ hr. during which time the contents of the tube were frequently well mixed, the copper-lime filtrate was obtained by centrifuging. This procedure was found to remove not only interfering carbohydrates but also protein, thus making further deproteinization unnecessary. The copper-lime filtrate (3 ml., containing 6-30μg. lactic acid) were added to a conical flask containing 0.15 ml. 4% copper sulphate solution. The lactic acid was converted to acetaldehyde by heating it with 18 ml. concentrated H_2SO_4 in a boiling water-bath for 5 minutes. After rapid cooling in ice 0.3 ml. of a 1.5% solution of p-hydroxy-diphenyl in 0.5% NaOH was added, and the flasks were immersed in a water-bath at 30°C for at least 30 minutes to hasten colour development. Excess reagent was finally destroyed by immersing the flasks in a boiling water-bath for 90 seconds and then

TABLE 1

Data obtained in order to calibrate the Spekker Absorptiometer for the estimation of lactic acid

Volume of solution taken for copper-lime treatment (ml.)	Concentration of lactic acid in copper-lime filtrate µg./ml.	Spekker reading (a)	Reading (a) - blank
Blank	0	0.023	
Soln. C. 2.5	1	0.068	0.045
" " 5.0	2	0.103	0.080
" " 7.5	3	0.143	0.120
" " 10.0	4	0.187	0.164
" " 12.5	5	0.220	0.197
Blank	0	0.040	
Soln. D. 7.5	6	0.280	0.240
" " 8.75	7	0.303	0.263
" " 10.0	8	0.358	0.318
" " 11.25	9	0.396	0.356
" " 12.5	10	0.430	0.390

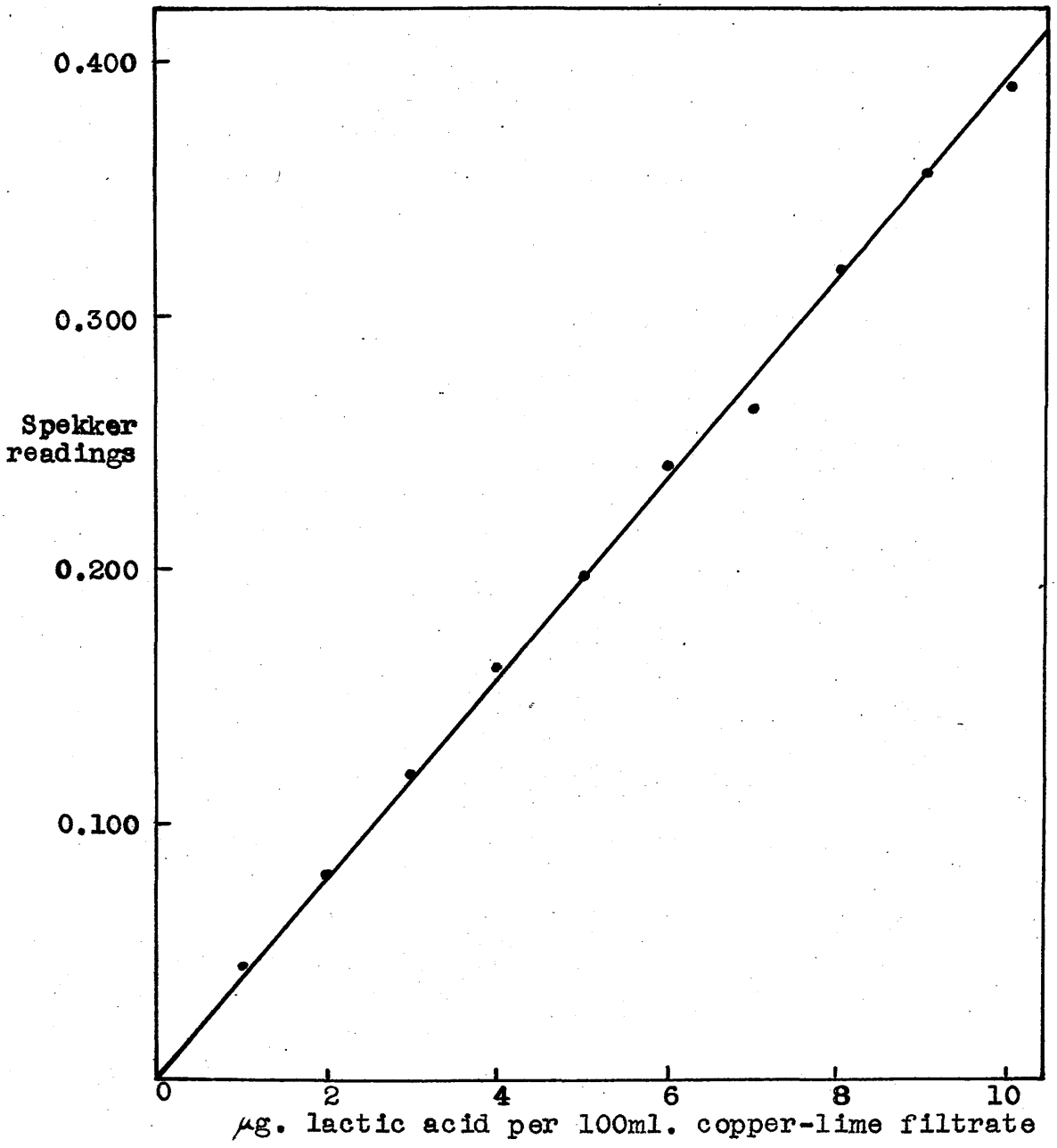


Figure 1. Calibration of Spekker Absorptiometer for lactic acid determination.

cooling in an ice-bath. The depth of colour developed in the solution was measured in a Spekker Absorptiometer using a green filter. A calibration curve for the instrument was constructed by using known amounts of lactic acid. A convenient method of doing this was to weigh accurately 1 g. lactic acid and dilute it to 1,000 ml. thereby obtaining a solution containing 1 mg./ml. This solution was then diluted 1 in 10 (solution B) and solution B diluted 1 in 10 and 1 in 5 (solutions C and D). Portions of these solutions as shown in Table 1 were then put through the copper-lime treatment and colour development procedure as already described. Blanks were similarly treated and the Spekker reading for the blank subtracted from the lactic acid Spekker readings before constructing the calibration curve shown in Figure 1.

Analysis of the Bacterial Sediment Obtained from Rumen Contents

Sediment weight and nitrogen content For routine analysis of the bacterial sediment, 40 ml. rumen liquid were pipetted into weighed 50 ml. centrifuge tubes and centrifuged for 45 minutes at 3,000 r.p.m. (centrifugal force = 1800 x g.). The supernatant layer was poured into a 100 ml. volumetric flask, and the sediment washed with about 25 ml. distilled water. After a further spinning the supernatant layer was added to the first. The washing was repeated a second time and the combined supernatant layers made up to the mark with water. The sediment was then washed twice with

alcohol and finally dried to constant weight at 100°C. It was then transferred quantitatively to a Kjeldahl flask with hot concentrated sulphuric acid for nitrogen determination. The liquid separated from the sediment was used for determining soluble carbohydrate.

Polysaccharide The method used was essentially that of the Association of Official Agricultural Chemists (1945), the procedure being as follows. A weighed amount of bacterial sediment (0.1 to 0.3 g.) was refluxed with 100 ml. H_2O and 10 ml. HCl (sp. gr.1.125) for 2½ hr. The reducing power of the hydrolysate was then determined as already described for hexose formed from maltose on p.21. The factor 0.9 was used to convert hexose to polysaccharide.

Pentose The method described by the Association of Official Agricultural Chemists (1945) for pentose was used. This method has already been described for determination of pentose in the separated rumen liquid. The distillation was begun with 2 g. of sediment and 100 ml. of 12% HCl.

Crude fibre Two methods were used, (a) the official method which is described fully by the Association of Official Agricultural Chemists (1945), and (b) a shorter method described by Whitehouse, Zarow and Shay (1945).

(a) Bacterial sediment (2 g.) was digested for ½ hr. with 1.25% H_2SO_4, and the unhydrolysable residue removed by passing the liquid through filter cloth. The residue was then digested for ½ hr. with 1.25% NaOH, and the residue from this digestion transferred to a Gooch crucible. After heating to constant weight at 110°C, the residue was incinerated. The loss of weight on incineration was attributed to fibre.

(b) Bacterial sediment (2 g.) was digested only once with 100 ml. of a reagent containing 20 g. trichloroacetic acid dissolved in a mixture consisting of 500 ml. glacial acetic acid, 450 ml. distilled water and 50 ml. nitric acid (sp. gr. 1.42). The residue was dried and incinerated as in (a).

Lipoid material An estimate of the lipoid material was made by heating 2 g. of the bacterial sediment with alcoholic KOH followed by extraction with ether. The ether was then evaporated and the residue extracted with light petroleum. The amount of lipoid material dissolved in the light petroleum was weighed.

Ash The sediment (1 g.) was incinerated in an open crucible at a dull red heat over a Bunsen burner till constant weight was attained.

Phosphorus The well-known method of Fiske and Subbarow (1925) was used. The solution for analysis was prepared by incinerating 2 g. bacterial sediment in the presence of added $MgCl_2$ to prevent loss of phosphorus, and dissolving the ash in HCl. The phosphate in the solution was first converted to phosphomolybdic acid, which was then reduced with 1, 2, 4 -aminonaphtho- sulphonic acid. The blue colour produced was measured in a Spekker Absorptiometer.

Calcium The well established method of McCrudden (1911-12) was adopted. The calcium in portions of the ash solution used for phosphorus determination was precipitated as oxalate and titrated against standard permanganate solution.

Part I. Experiments on the Value of *in vitro* Studies, and on the Decomposition of Carbohydrate by Rumen Liquid

Introduction

It has already been explained that in earlier work when Pearson & Smith (1943, 1) attempted to study the utilization of N.P.N. by *in vivo* experiments with a bullock having a rumen fistula, they encountered great difficulty in obtaining from the heterogeneous mass of rumen contents, samples which were sufficiently representative and homogeneous for chemical analysis. They also foresaw difficulty in interpreting the results which might be obtained, since it would be difficult to know accurately how far a decrease in the N.P.N. content of the rumen ingesta could be attributed to conversion to protein and how much to its absorption from the rumen into the blood or to its passage further along the alimentary tract. They therefore adopted a method whereby rumen contents removed by way of the fistula were strained through muslin and incubated in the laboratory according to the method which has been described on p. 18.

It was clear, however, that conclusions drawn from *in vitro* incubations of this type might well be criticised on the grounds that they were not necessarily applicable to the normal rumen where saliva is constantly being added to the ingesta and where there is selective absorption from the rumen to the blood and

passage of rumen contents to the other stomachs and intestines. It was decided, therefore, that before further *in vitro* work was undertaken, tests should be made to determine whether results obtained by this technique were likely to be applicable to the normal rumen.

It is well known that CO_2, CH_4, lower fatty acids and lactic acid are formed in the rumen when carbohydrate is fermented, and recently Phillipson (1942) has shown that the production of these substances reaches a maximum during the first few hours after feeding, the changes being accompanied by a decrease in pH. Since in the *in vitro* studies of Pearson & Smith (1943, 3) and Smith & Baker (1944), starch or maltose was added to the rumen liquid immediately before incubation began, the changes occurring in the incubation period might be expected to bear some resemblance to those observed in the rumen during the first few hours after feeding. Should there be a similarity between *in vivo* and *in vitro* fermentation of carbohydrate, it would be reasonable to suppose that the nitrogen changes observed in the *in vitro* work, would also resemble those which take place in the intact animal.

The experiments carried out to determine the value of *in vitro* work led also to another consideration. In a constantly changing system like the rumen from which absorption direct into the blood and passage further along the alimentary tract can occur, it is

extremely difficult to determine how much of the carbohydrate fermented in a given time is converted to lower fatty acids and how much to other products such as CO_2, CH_4, lactic acid, protein and the bacterial starch - like polysaccharide which has been discussed by Baker (1943) and Baker and Harriss (1947) and estimated in bacterial sediment by Smith & Baker (1944). In the work described in this section, therefore, the opportunity was taken of using the in vitro technique to find how much of the sugar which was decomposed in a given time could be accounted for by the formation of these various products.

There were, therefore, two objects, first to determine whether the results obtained by in vitro incubation could reasonably be applied to the normal rumen, and second to determine how much of the fermented carbohydrate could be accounted for by the various products formed.

Experimental

(1) A preliminary experiment on the formation of lower fatty acids and lactic acid during in vitro incubation The aim of this experiment was to determine whether in incubations, in which protein synthesis occurred, the fermentation of carbohydrate was accompanied by the formation of lower fatty acids and lactic acid. Rumen liquid (2,000 ml.) which had been strained through muslin, but not centrifuged, was

TABLE 2

The formation of volatile fatty acids and lactic acid from maltose during the incubation of rumen liquid

Hours	pH	Maltose	Volatile fatty acids (as acetic acid)	Lactic acid
		mg. per 100 g. rumen liquid		
0	6.0	1005	636	21.4
0.5	6.1	909	690	37.1
1.0	5.8	787	712	49.9
1.5	5.6	676	748	57.8
2.0	5.6	578	777	71.2
3.0	5.4	398	844	79.2
4.0	5.3	296	902	94.2

incubated for 4 hours in the presence of 1% maltose. Urea (0.05%) was also added to ensure that bacterial growth was not limited by lack of N.P.N., since the naturally occurring N.P.N. content of rumen liquid is sometimes low. Initially and at intervals during the incubation period samples of the liquid were removed for the determination of pH and for the estimation of soluble sugar, lower fatty acids and lactic acid.

From the results which are recorded in Table 2 and Fig. 2 it can be seen that the fermentation of carbohydrate was accompanied by the production of lower fatty acids and lactic acid and that after the first half-hour* there was a decrease in pH. These changes are qualitatively similar to those which Phillipson (1942) detected during the fermentation of carbohydrate in vivo, and it was clear that further experiments should be made in which the formation of CO_2 could be studied, since the production of CO_2 is also one of the characteristics of carbohydrate fermentation in the rumen.

(2) CO_2 production during in vitro incubation Two experiments were carried out in which 900 ml. of rumen liquid containing 1% maltose and a small amount of urea were incubated for 4 hours in a flask fitted in such a way that a slow current of CO_2-free air could be drawn through the liquid and then passed through bottles

* The slight increase in pH during the first half-hour resulted from the rapid conversion of urea to ammonia during that period (Pearson & Smith, 1943, 2).

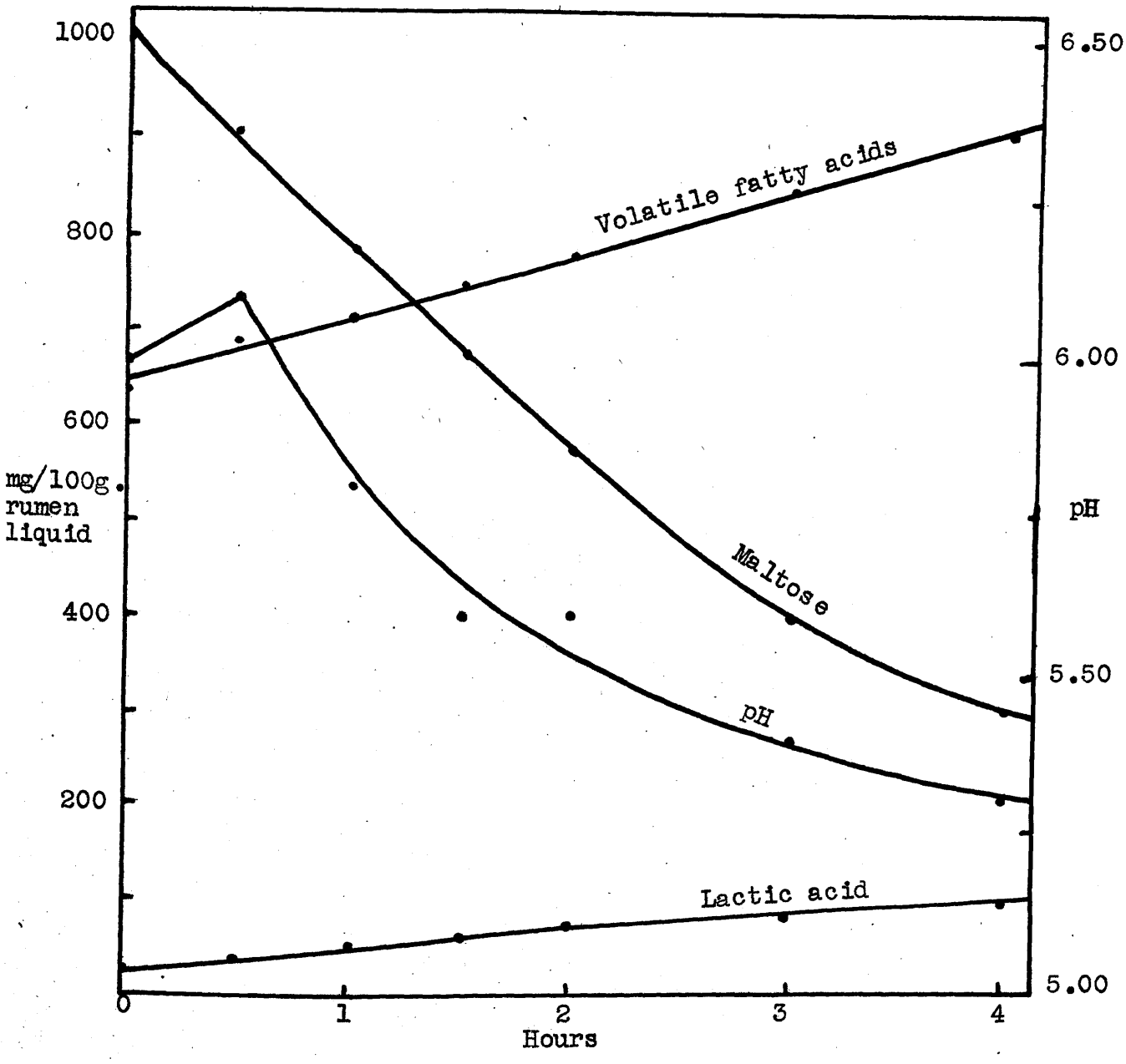


Figure 2. The formation of volatile fatty acids and lactic acid from maltose during the incubation of rumen liquid.

Note: The initial increase in pH was due to formation of ammonia from urea.

TABLE 3

The formation of carbon dioxide in two typical incubations of rumen liquid containing maltose and urea

	Experiment 1	Experiment 2
	mg./100 g. rumen liquid	
(a) CO_2 collected in baryta	157.0	132.0
(b) CO_2 calculated to have come from urea	50.6	30.6
(c) CO_2 derived mainly or entirely from maltose (a - b)	106.4	101.4
(d) The carbon equivalent of the CO_2 (c x 12/44)	29.0	27.7
(e) Decrease in the maltose content of the rumen liquid during incubation	706.0	619.0
(f) The carbon equivalent of the maltose (e x 144/342)	297.5	260.8
Approximate amount of maltose converted to CO_2	9.8%	10.6%

containing standard baryta. At the end of the incubation, the first two bottles of baryta showed some turbidity, indicating that they had trapped CO_2 whereas the liquid in the third bottle was still transparent. The baryta in these first three bottles was titrated with standard HCl using thymolphthalein as indicator (Martin & Green, 1933) and in this way an approximate estimate of the CO_2 formed during the incubation was obtained. It was realized from the work of Pearson & Smith (1943, 2) that the urea which had been added would be hydrolysed to ammonia and CO_2 during the incubation and so a correction was made for the amount of CO_2 which would be produced in this way. Since the pH decreased by nearly one unit during the two incubation periods, a small proportion of the CO_2 which was collected was probably present in the rumen liquid before incubation and was liberated as a result of the medium becoming slightly more acid. This small proportion would not be formed from maltose, and so a correction for it would have to be applied if an accurate result had been desired, but since these experiments were of a preliminary nature, the correction was not made in this particular instance.

The results of these two experiments are given in Table 3 and show that probably about 10% of the carbohydrate fermented was converted to CO_2. This value cannot be other than approximate, but it suggests that the changes which had taken place in the incubation flask were similar to those which are known

to occur when carbohydrate is fermented *in vivo*, for it has been stated that in cellulose digestion under natural conditions about 7% is converted to gas, (Hammond 1928). Further experiments were now planned to obtain a more exact measure of the CO_2 produced and at the same time to estimate the other products of fermentation, and so determine what proportion of the fermented carbohydrate may be accounted for by the various end-products.

(3) *The amount of carbohydrate fermented in vitro which can be accounted for by the production of the various end-products* Since the preliminary experiments, which have just been described, indicated that the changes which occurred *in vitro* were probably very similar to those which occurred *in vivo*, particularly after a meal, it seemed that these *in vitro* methods might give valuable information regarding the proportion of the fermented carbohydrate which is converted to each of the various fermentation products. In a typical experiment designed with this object in view, sufficient rumen contents were strained through muslin and centrifuged to give 2 litres of liquid. Maltose (1%) and urea (0.05%) were added, and the liquid divided into two portions. Samples of one portion, preserved by adding a known amount of 10N H_2SO_4, were kept for the determination of lower fatty acids, lactic acid, maltose, readily hydrolysable polysaccharide and protein. A further

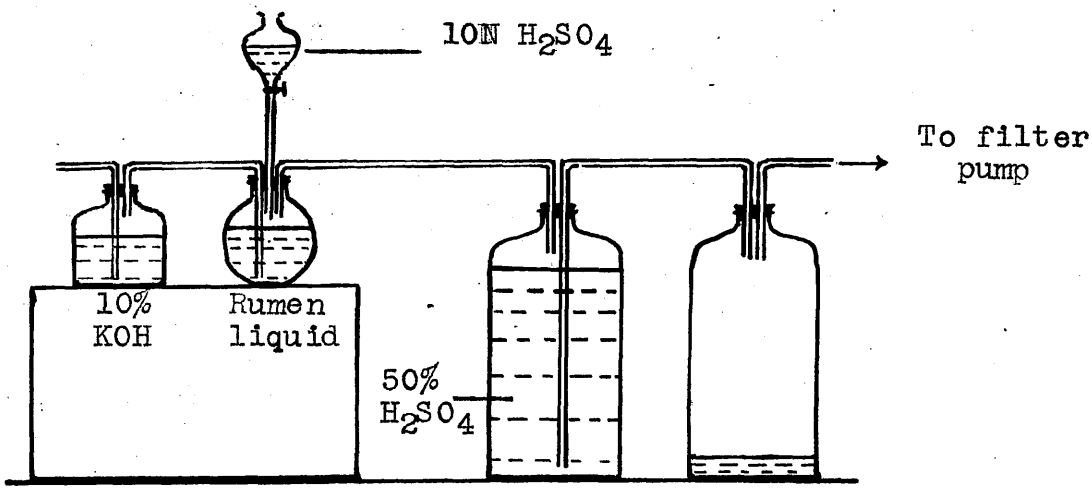


Figure 3a. Apparatus for acidifying the rumen liquid and collecting the gas evolved.

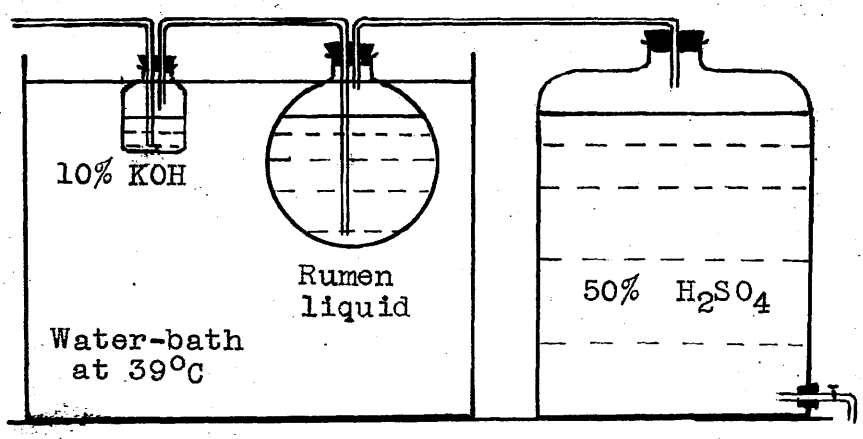


Figure 3b. Apparatus for collecting the gas from rumen liquid during incubation.

sample of the same portion (200 ml.) was placed in a flask arranged as shown in Fig.3a. The flask was closed by a bung, fitted with a tap-funnel and two glass tubes, one of which extended below the surface of the liquid inside the flask and was attached at its other end to a CO_2-trap through which atmospheric air could be drawn. The other tube did not extend below the surface of the liquid in the flask and was connected with a Winchester bottle filled with 50% H_2SO_4. A current of CO_2-free air was drawn through the rumen liquid in the flask by withdrawing the acid from the Winchester bottle in which the air and gases from the rumen liquid were collected. H_2SO_4 (50%) was used rather than water to prevent solution of CO_2. Just as the stream of air began to pass through this particular sample of rumen liquid, the liquid was acidified with 40 ml. 10N H_2SO_4 from the tap-funnel. By measuring the volume of gas collected, (about 2 litres in a typical experiment) and analysing it in a Haldane gas-analysis apparatus, the amounts of CO_2 and CH_4 removed from the acidified rumen liquid before incubation were obtained.

Immediately after dividing the original rumen liquid into two portions, the main portion (500 ml.) was incubated for 4 hours at 39°C in a flask fitted with the type of gas-collecting apparatus shown in Fig.3b. During the incubation period a slow current of CO_2-free air was drawn through the liquid and the

gases collected in the aspirator. At the end of the period, some of the rumen liquid was drawn off and preserved for the determination of lower fatty acids, lactic acid and other products of fermentation. At the same time, 200 ml. of the liquid which had been incubated was transferred to the gas-collection apparatus (Fig.3a), acidified with 10N H_2SO_4 and a rapid current of CO_2-free air passed through it so that any CO_2 and CH_4 still remaining in it could be estimated.

Since combustible gases such as olefines and carbon monoxide might have been produced during some types of fermentation and since the presence of very small proportions of carbon monoxide have sometimes been reported in rumen gases, (Olsen, 1942; Dougherty, 1941), samples of the gas collected during incubation were treated with bromine water and with cuprous chloride in a Orsat-Fischer gas-analysis apparatus. No traces of olefines or carbon monoxide were detected. On combusting the gas collected from the incubation in the Haldane apparatus, it was found that the amount of contraction which occurred during combustion was twice the volume of the CO_2 produced. This confirmed that the only combustible gas likely to be present in significant amounts was methane, since the equation for the combustion of methane is $CH_4 + 2O_2 = CO_2 + 2H_2O$, from which it can be seen that the volume of oxygen used is twice that of the CO_2 produced.

TABLE 4

Typical gas-analysis data obtained in order to estimate the amount of carbon dioxide and methane produced during incubation

	(a) The gas obtained during incubation		(b) The gas obtained by aerating an acidified sample of unincubated rumen liquid		(c) The gas obtained by aerating a sample of rumen liquid acidified after incubation	
	Haldane data (ml.)	Composition (%)	Haldane data (ml.)	Composition (%)	Haldane data (ml.)	Composition (%)
Volume of sample taken in Haldane burette	8.028		7.837		7.798	
" " CO_2 in the sample	0.338	4.21	0.100	1.28	0.052	0.67
" " contraction after combustion	0.165		0.007		None	
" " CO_2 formed on combustion	0.083		0.003		None	
" " contraction + CO_2 formed on further combustion	0.039		0.004		None	
Total volume of contraction + volume of CO_2 formed on combustion (v)	0.287		0.014		None	
Volume of methane (v/3)*	0.096	1.20	0.005	0.06	None	None
Volume of residual oxygen	1.371	17.08	1.578	20.13	1.594	20.45
" " " nitrogen	6.032	75.13	6.148	78.45	6.152	78.90
" " oxygen used in combustion of methane		2.40		0.12		None
		100.02		100.04		100.02
Average of 3 analyses, carbon dioxide (%)		4.26		1.25		0.68
" " " " methane (%)		1.23		0.06		None

* $CH_4 + 2O_2 = CO_2 + 2H_2O$

Note: The volume of the contraction in the first combustion was twice the volume of CO_2 produced, indicating that the combustible gas almost certainly consisted entirely of methane.

TABLE 5

The calculation of the amount of carbon dioxide and methane produced during a typical incubation of rumen liquid using the gas-analysis results obtained in Table 4

	(a) The gas obtained during incubation	(b) The gas obtained by aerating an acidified sample of unincubated rumen liquid	(c) The gas obtained by aerating a sample of rumen liquid acidified after incubation
Volume of gas collected at N.T.P.	7650 ml.	2120 ml.	2198 ml.
Weight of rumen liquid from which the gas was derived	500 g.	200 g.	200 g.
Volume of gas/100 g. rumen liquid	1530 ml.	1060 ml.	1099 ml.
Carbon dioxide in the gas (obtained from Table 3)	4.26 %	1.25 %	0.68 %
Volume of CO_2 in ml./100g.rumen liquid	65.33	13.25	7.47
Weight of CO_2 in mg./100g.rumen liquid	128.3	26.0	14.7
Methane in the gas obtained from Table 3	1.23 %	0.06 %	None
Volume of methane in ml./100 g. rumen liquid	18.82	0.64	None
Weight of methane in mg./100 g. rumen liquid	13.44	0.45	None

Corrections : CO_2 derived from hydrolysis of 50 mg.urea = 36.7 mg./100 g. rumen liquid

CO_2 liberated during incubation from carbonates or bicarbonates as a result of a decrease in pH = 26.0 - 14.7

= 11.3 mg./100 g. rumen liquid

CO_2 derived from maltose = 128.3 - (36.7 + 11.3)

= 80.3 mg./100 g. rumen liquid

Methane originally present = 0.45 mg.

Methane derived from maltose = 13.0 mg./100 g. rumen liquid

The results for three typical experiments will now be discussed. The amounts of the various end-products, other than gases, were determined by analysis of the rumen liquid before and after incubation, using the methods which have already been described (pp. 19 - 27). The gases obtained from the rumen liquid acidified before incubation, from the rumen liquid acidified after incubation and from the unacidified sample during incubation were measured and samples analysed on the Haldane apparatus.

Typical results obtained in one experiment for the gas-analysis are shown in Table 4. It will be observed from these results that CO_2 and CH_4 were originally present in the rumen liquid (Column b, Table 4) and that they were both formed during incubation (Column a), but that no methane was contained in the rumen liquid after incubation (Column c), all measurable amounts of it having been collected before incubation ceased. Each gas analysis was done in triplicate and the results for the individual analyses agreed very closely indeed. For calculating the amounts of CO_2 and CH_4 formed, the average results of the three analyses were used. These average results are recorded at the end of Table 4.

The method of calculating the amounts of CO_2 and CH_4 formed during incubation is shown in Table 5. There were originally 50 mg. urea present in 100 g. rumen liquid, and during incubation the pH changed

TABLE 6

The amounts of the various products formed in three separate incubation experiments with maltose

	Amount of substance decomposed or formed during incubation (mg./100 g. rumen liquid)		
	Experiment No.		
	1	2	3
Maltose changed during incubation	583	445	550
Lower fatty acids produced (as acetic acid)	170	85	201
Lactic acid produced	34	44	49
Carbon dioxide "	66	47	80
Methane "	11	4	13
Bacterial protein* produced	31	53	60
Bacterial polysaccharide produced	172	161	77
pH changes, Initial	5.80	5.94	6.54
Final	5.39	5.40	5.56
Change	-0.41	-0.54	-0.98

* 6.25 x the decrease in N.P.N.

TABLE 7

The amount of maltose, expressed in terms of carbon, which was utilized during incubation and which was accounted for by the carbon of the various end-products formed in three typical experiments

	Amount formed during incubation (mg.carbon/ 100g.rumen liquid)			The percentage of the maltose carbon accounted for by each product		
	Experiment No.			Experiment No.		
	1	2	3	1	2	3
Carbon of:-						
Volatile fatty acids (as acetic acid)	68	34	80	27.6	18.2	34.5
Lactic acid	14	18	20	5.7	9.6	8.6
Carbon dioxide	18	13	22	7.3	7.0	9.5
Methane	8	3	10	3.3	1.6	4.3
Bacterial protein	16	28	32	6.5	15.0	13.8
Bacterial polysaccharide	76	72	34	30.9	38.5	14.7
Total	200	168	198	81.3	89.9	85.4
Carbon of maltose, changed during incubation	246	187	232	100	100	100
Proportion of maltose accounted for (%)	81.3	89.9	85.4	81.3	89.9	85.4
If the volatile fatty acids are calculated as propionic acid instead of acetic acid, the proportion of maltose carbon accounted for would be				95.1	98.9	102.6

from 6.54 to 5.56. Corrections were made, therefore, for the CO_2 which would arise from urea and also for the amount which would be liberated from the natural buffer systems of the rumen liquid as the acidity of the medium increased.

The results for CO_2 and CH_4 arrived at in this way for three quite distinct but typical experiments are shown in Table 6, together with the results for the other end-products and the changes which occurred in pH.

In order to determine how much of the utilized maltose could be accounted for by the formation of these various products, the figures tabulated in Table 6 had to be converted to equivalent amounts of carbon. By this means it was possible to calculate the total amount of carbon in the end-products and compare it with the amount of carbon which was contained in the utilized maltose. The results of these calculations which are recorded in Table 7 suggest that about one third to one half of the maltose utilized during incubation was converted to bacterial protein and bacterial polysaccharide, the remainder being fermented. Assuming that the volatile fatty acids consisted entirely of acetic acid, the results in Table 7 indicate that about 81 to 90% of the maltose utilized would be accounted for. It is well known, however, that propionic acid and a small amount of butyric acid are usually present in the volatile fatty acids formed in the rumen, but estimates vary as

TABLE 8

The proportion of the fermented maltose, expressed as carbon, which was accounted for by the carbon of the various end-products of fermentation

	The percentage of the maltose carbon utilized during incubation and accounted for by the four end-products of fermentation			The percentage of the maltose assumed+ to be fermented accounted for by each product, calculated on the basis of carbon		
	Experiment No.			Experiment No.		
	1	2	3	1	2	3
The carbon of:-						
Volatile fatty acids (as acetic acid)	27.6	18.2	34.5	44.1	39.1	48.2
Lactic acid	5.7	9.6	8.6	9.1	20.7	12.1
Carbon dioxide	7.3	7.0	9.5	11.7	15.1	13.3
Methane	3.3	1.6	4.3	5.3	3.4	6.0
Unaccounted for	18.7	10.1	14.6	29.8	21.7	20.4
Total	62.6	46.5	71.5	100.0	100.0	100.0

* The values in these first three columns are taken from Table 7.

\+ Namely, the total maltose changed during the incubation less the amount utilized for the formation of bacterial protein and polysaccharide.

as to the actual proportions in which each occurs, (Phillipson, 1947-48; Elsden, 1945-46). Further experiments are now being carried out by the present writer to determine the precise nature of the mixture of volatile acids which are formed during *in vitro* incubations of this type but the work has not yet advanced sufficiently far to be described. It can be seen from the figures in the last line of Table 7 that if the main acid were propionic rather than acetic, considerably more of the utilized maltose would be accounted for, the percentage recovery figures in that case being 95.1, 98.9 and 102.6. If, however, an equimolecular mixture of acetic and propionic acids were produced, the percentage recovery figures would be 88.2, 94.4 and 94.0. In work of this type recoveries in the order of 95% would clearly suggest that all the end-products of fermentation were being estimated.

The figures recorded in the last three columns of Table 7 for the end-products of fermentation as distinct from the formation of bacterial protein and polysaccharide have been used in Table 8 to calculate the proportion of the fermented maltose which could be accounted for by each of the four products. Assuming that the main volatile acid was acetic, about half of the maltose fermented was converted to volatile fatty acids, a tenth to a fifth was converted to lactic acid and almost the same amount to CO_2, but only about 3 to 6% to methane. Of the total maltose utilized during

incubation (Table 7) 10 to 19% was unaccounted for, and if this is assumed to have been fermented maltose, it means that about 20 to 30% (Table 8) of the fermented maltose was converted to some product other than the four which have been studied. If, however, propionic and acetic acids were produced in equivalent amounts, the amount of fermented maltose unaccounted for would be reduced to about 8 - 18% and the amount accounted for by the formation of volatile fatty acids increased to 55 - 60%. As already explained further work on this aspect of the subject is now in progress.

So far it has been assumed that the various substances produced during these incubations were formed from the maltose which was added to the rumen liquid before incubation began, but it may be that some of these products can be formed in significant amounts from other constituents of the rumen liquid during an incubation period of 4 hours. To investigate this point two separate experiments were carried out in which rumen liquid was incubated under the same conditions as in the experiments already described except that no maltose was added. The various products formed during incubation were estimated exactly as shown in Tables 4 to 7 for the incubations in which maltose was present. Since the method has already been so fully described, only the final results need be given. They are recorded in Table 9 and should be compared with the corresponding values in Table 6 and in the first three columns of Table 7. It will be noted that the amounts

TABLE 9

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

	The amount of substance decomposed (-) or formed (+) during incubation (mg. substance/ 100g. rumen liquid)		The amount of substance decomposed (-) or formed (+) expressed as carbon (mg. carbon/ 100g. rumen liquid)	
	Experiment No.		Experiment No.	
	1	2	1	2
Volatile fatty acids (as acetic acid)	+ 9.0	+26.0	+ 3.6	+10.4
Lactic acid	None	+ 0.2	None	+ 0.1
Carbon dioxide	+ 8.9	None	+ 2.4	None
Methane	+ 0.5	+ 0.8	+ 0.4	+ 0.6
Bacterial polysaccharide	+ 1.0	- 0.5	+ 0.4	- 0.2
Soluble carbohydrate	- 8.9	- 2.3	- 3.6	- 0.9
(a) Bacterial protein*	-16.5	-15.6	- 8.8	- 8.3
(b) Total of substances decomposed expressed as carbon			-12.4	- 9.4
Proportion of total decomposition which can be attributed to protein breakdown $\frac{(a \times 100)}{b}$			71.0%	88.3%
pH changes: Initial	6.3	6.8		
Final	7.1	7.8		
Change	+ 0.8	+ 1.0		

* The increase in N.P.N. x 6.25

Note. The results in the first two columns for these two experiments without added maltose should be compared with the corresponding results recorded in Table 6 for three incubations in the presence of 1% maltose. Similarly the results in the last two columns above should be compared with those in the first three columns of Table 7.

of the various substances formed in 4 hours in the absence of added carbohydrate were very much smaller than the corresponding values when maltose was present. In one of the two experiments there was only a small amount of volatile fatty acid formed and no lactic acid. In the other there was only a trace of lactic acid and no measurable amount of carbon dioxide. In both there was a small loss of soluble carbohydrate, but according to the increase which occurred in the N.P.N. content of the rumen liquid, the main substance decomposed was protein. This decomposition of protein in the absence of added carbohydrate confirms the earlier work of Pearson & Smith (1943, 3) in which they found that protein synthesis occurred only when there was a supply of readily available carbohydrate in the rumen liquid. In fact it can be seen from Table 9 that 71 and 88% of the total decomposition might reasonably be attributed to protein. In experiments of this type in which only very small amounts of the various products are formed, it is not easy to estimate them with great accuracy. However, the results are sufficiently reliable to show that by far the greater part of the small amounts of volatile fatty acids, carbon dioxide and methane which were formed in these two incubations were derived from the decomposition of protein. The assumption may safely be made, therefore, that in incubations in which a soluble sugar such as maltose is added and in which protein synthesis predominates over protein breakdown, the various products formed are derived almost entirely from the added sugar.

Discussion

One of the two main objects of the foregoing experiments was to determine whether the changes occurring during *in vitro* incubations of rumen liquid in the presence of added carbohydrate were similar to those which occur in the normal rumen during the hours which follow the ingestion of food. The various items studied included change in pH and the formation of CO_2, CH_4, lactic acid and volatile fatty acids.

The pH changes occurring during *in vitro* incubations in the presence of added carbohydrate when protein was being synthesised are shown in Table 6, and typical changes in the absence of added carbohydrate in Table 9. When soluble carbohydrate was present, the pH frequently decreased by as much as one unit, often reaching a final value of 5.4, but when no carbohydrate was added there was always an increase of up to one unit. This increase was doubtless due to the decomposition of protein and the formation of ammonia which occurs under these circumstances. A brief survey of the published observations on this subject discloses that the normal range of the pH of rumen contents *in vivo* is large. Monroe and Perkins (1939) reported average values of between 6.8 and 7.0 for samples of rumen ingesta obtained from fistula animals receiving hay and concentrates and lower values of 6.5 and 6.7 for cattle at pasture. In general, during the 4 hours following a meal, the pH decreased considerably, sometimes by as much as one unit and then

tended to rise slowly. Hale, Duncan and Huffman (1940) have studied the pH changes which occur throughout the day and have also related these changes to times of feeding. Again there was a marked decrease for some hours after food, followed by a slow rise. Later work by Phillipson (1942) showed that pH changes occurring in the rumen of sheep after different types of feed, were similar to those demonstrated for the bovine rumen. Phillipson also confirmed that the pH was considerably lower when sheep were fed grass, the average variation being from 6.39 prior to feeding to 5.80 at the peak of fermentation. Some sheep, however, developed a pH as low as 5.4. Myburgh & Quin (1943), also with sheep, found that the pH of rumen ingesta varied from 5.5 to 6.8, depending on the carbohydrate of the diet.

It appears, therefore, that pH values found for rumen ingesta in vivo may vary between 7.1 and 5.4 depending largely on the type of diet and the time of sampling after feeding. Thus the pH values recorded in the present in vitro experiments with added carbohydrate are within the normal range. Furthermore, Phillipson and McAnally (1942) have found that when 100 g. of galactose, a sugar which behaves similarly to maltose in the rumen, is given to a sheep, the pH changes from 6.7 to 6.2 within $4\frac{1}{2}$ hours. Thus the pH changes which were found in vitro closely resemble those reported for in vivo digestion of soluble sugar. From a consideration of pH changes alone, therefore, it would appear that the changes taking place during the short

in vitro incubations with added sugar closely resemble those which take place in the rumen some hours after a meal.

Phillipson and McAnally studied the formation of volatile fatty acids and lactic acid which accompanied these changes in pH. In the *in vivo* experiment with galactose to which reference has just been made, it was estimated that there had been an increase in volatile fatty acids (calculated as acetic acid) of 170 mg. per 100g. rumen liquid within 4½ hours, a figure similar in order of magnitude to those of 170 and 201 found *in vitro* and recorded in Table 6. In the *in vitro* incubations, lactic acid was produced in much smaller quantities than the volatile fatty acids, and this again is in accordance with Phillipson's observation that there was very little accumulation of lactic acid *in vivo*. In the living animal, lactic acid formation might appear to be low because it might leave the rumen rapidly either by preferential absorption into the blood stream or by passing further along the alimentary tract, but the *in vitro* work described here suggests that with some samples of rumen liquid relatively little is actually formed.

Various workers have studied the composition and quantity of the fermentation gases produced in the rumen. The ratio of CO_2 to CH_4 has been largely investigated owing to the fact that this ratio is used to obtain a true value for expired CO_2 in respiratory quotient determinations on cattle. The work of

Washburn and Brody (1937) showed conclusively, however, that the ratio of carbon dioxide to combustible gas was not a constant, but varied with the time after feeding, the ratio rising with volatile fatty acid production and consequent liberation of carbon dioxide from carbonates. In the present experiments, the ratio of the volume of CO_2 to that of CH_4 produced during four hours' incubation in Experiments 1, 2, and 3, and recorded in Table 6, may be calculated to be 2.18, 4.27 and 2.24. When it is considered that Kleiber, Cole and Mead (1943) reported an average value of 2.58 for this ratio and that for the rumen gas of a Holstein cow slaughtered four hours after a feed of alfalfa hay, silage and grain mixture, Washburn and Brody (1937) found the ratio to be 2.40, it may be concluded that production in vitro follows the same trend as in vivo.

It is difficult to obtain information from the literature concerning the amount of methane produced per unit of carbohydrate fermented. Olsen (1940) quotes some figures among which is that of Kellner's of 3.17 g. CH_4 produced per 100g. starch digested. From the amounts recorded in Table 6 it can be calculated that in the present in vitro incubations the amount of methane produced was equivalent to 1.89, 0.9 and 2.36 g. methane per 100 g. of sugar fermented. In view of the very different circumstances in which they were determined these figures are close enough to those of Kellner to support the conclusion that as far as gas production is concerned, fermentation in vitro is

analagous to that *in vivo*.

Elsden (1945-46) has published results of both *in vitro* and *in vivo* experiments on the fermentation of carbohydrate in the rumen of sheep. It is of interest to note that in his *in vivo* work he found that the dietary history of the animal had a profound effect on the rate of fermentation of glucose. In one experiment the dose of glucose had not disappeared at the end of ten hours, whereas in another experiment two hours sufficed for its disappearance. The rate of lactic acid and volatile fatty acid production differed in a similar way. If changes at the end of the fourth hour only had been studied, the results for lactic and volatile fatty acids would probably have varied from experiment to experiment in much the same way as they varied in the present *in vitro* studies.

These considerations lead to the conclusion that in the *in vitro* technique of Pearson and Smith (1943, 3) which has been used in the present work, the changes occurring when soluble carbohydrate is added to the rumen liquid are very similar to those occurring in the rumen itself during a period of several hours following a meal. It is probable, therefore, that conclusions regarding protein synthesis obtained in the *in vitro* work can reasonably be applied to the intact rumen.

The second object of this part of the work was to determine how far the sugar utilized in an incubation could be accounted for by the formation of

bacterial protein, polysaccharide, CO_2, CH_4, lactic acid and volatile fatty acids. Table 7 shows that some 81 to 90% of the sugar could be accounted for by the formation of these products. The fact that 10 to 19% remained unaccounted for may be attributed to three causes. First, it is quite possible that when sugar is fermented by rumen contents, small amounts of products are formed other than those which have been studied here. Further work is required to explore this possibility. Secondly, in estimating the various products listed in Table 6, there were bound to be small experimental errors which might be accumulative. It is not easy to obtain definite information about this. Thirdly, for calculation purposes, the volatile fatty acids were assumed to be acetic acid, although it is realized that they also contained some propionic and possibly also some butyric acid. Elsden (1945-46) used an *in vitro* technique by which he found that glucose gave a mixture of volatile fatty acids consisting largely of propionic acid, whereas dried grass gave a mixture in which acetic acid predominated. Elsden's technique was to add the substrate such as glucose or cellulose to an inorganic medium resembling sheep's saliva which was then inoculated with rumen liquid and incubated for several days. Under these circumstances it could not readily be assumed that the surviving bacteria were truly representative of the mixed rumen flora, since it is well known that most of the bacterial strains present in the rumen do not readily survive for

more than a few hours in artificial media, and this would probably have an important bearing on the type of fatty acids produced. Obviously further work on the production of volatile fatty acids is required, using the *in vitro* technique described in the present work, in which rumen liquid itself, and not an inorganic medium, is incubated for only a few hours, a period during which it bears a close resemblance to normal rumen contents. Work along these lines is at present in progress.

Conclusion

From the evidence which has just been presented, it is concluded that the fermentation of maltose during *in vitro* incubation is essentially similar to the fermentation of soluble carbohydrate which occurs *in vivo* after ingestion of food, and since the fermentation of carbohydrate in the incubation flask is accompanied by protein synthesis, it is reasonable to conclude that protein synthesis also occurs in the intact rumen, particularly in the period which follows a meal.

Of the maltose decomposed during rumen digestion *in vitro* at least 80 - 90% can be accounted for by the formation of bacterial polysaccharide, bacterial protein, volatile fatty acids, lactic acid, carbon dioxide and methane.

Part II. The Preparation of a Dried Sample of Rumen Bacteria and the Biological Value of its Protein

Introduction

The work described by Pearson and Smith (1943, 3) and by Smith and Baker (1944) confirmed by *in vitro* methods that protein can be formed in rumen liquid from N.P.N. under certain circumstances, one of the main factors promoting synthesis being the presence of readily available carbohydrate. But once the possibility of protein synthesis had been established it was important to know something of the nutritive value of the protein so formed. Probably the earliest attempt to investigate this was made by Müller (1906), who inoculated culture medium with rumen bacteria, and after allowing growth to proceed for 3 days, precipitated the mixture with ammonium sulphate and fed a bitch with the dried precipitate. He found that nitrogen retention on the ration containing the bacterial protein was similar to that on rations containing albumin and casein. More recently Usuelli and Fiorini (1938) made preparations of rumen bacteria, of rumen protozoa and of residues of feedingstuffs from the rumen, and gave them as supplements to a basal diet in growth experiments with chickens. The basal diet supplemented with the residues of feedingstuffs was not significantly better in promoting growth than the basal diet alone, but the bacterial and protozoan preparations

brought about more rapid growth.

A further endeavour has been made to determine the nutritive value of the bacterial protein of the rumen by Johnson, Hamilton, Robinson and Garey (1944) who made dried preparations of rumen bacteria, of protozoa and also of an organism which they isolated in pure culture from the mixed rumen flora. Unfortunately only about 80 g. of each preparation was available with the result that only 2 or 3 rats could be used in the tests with each preparation, and the results obtained for the individual rats varied greatly. Thus the three results for the biological value of the protein in the mixed rumen preparation were 56, 70 and 73. Moreover, the mixed bacterial preparation had a fibre content which was as high as 1.4%, so that it was probably seriously contaminated with feedingstuffs residues. It was clear, therefore, that further work was required to determine more accurately the nutritive value of the rumen microorganisms and so experiments were planned in which reasonably large amounts of dried bacteria and of dried protozoa would be prepared and their nutritive values tested. The work on the protozoa is still in its preliminary stages, but the section on the bacteria has been completed and will now be described.

Experimental

The preparation of dried rumen bacteria The fact was

realised at the outset that the work involved in preparing a few hundred grams of dried bacteria would take several months and that a very large volume of rumen liquid would have to be dealt with. It was obvious, therefore, that the procedure adopted for preparing the bacteria would have to be chosen so as to give the maximum yields of bacterial protein which could conveniently be obtained. There were several possibilities. The animal could either be kept at grass, or could be stall-fed; the bacteria could be separated from the rumen liquid either immediately the liquid was removed from the rumen or after incubation in vitro with added carbohydrate.

In some preliminary tests it was found that by incubating the rumen liquid with maltose before isolating the bacteria, the weight of dried bacteria obtained was increased by nearly 50% and the amount of bacterial protein by 34%. It was decided, therefore, to incubate the rumen liquid before making the bacterial preparation. In the preliminary tests it was found that when the animal was grazing higher yields of the bacterial preparation could be obtained than when it was stall-fed, but owing to the length of time which was obviously going to be necessary to complete the work, it was not practicable to keep the animal at pasture for the duration of the experiment. It was, therefore, decided to maintain the animal on indoor rations. Throughout these particular experiments, the animal was fed a diet consisting of 16 lb. hay, 2 lb. bean meal,

2 lb. oats, 2 lb. dried grass, 2 lb. beet pulp and 15 lb. cabbage per day.

Over 400 g. of dried bacteria were prepared in small batches at a time over a period of 3 or 4 months. In a typical experiment 3 litres of rumen contents were removed from the rumen between 1 and 2 hours after feeding, strained through muslin and cleared of protozoa and finely divided vegetable material by centrifuging as described on p.18. Maltose (1%) and urea (0.05%) were then added to the liquid and a sample taken for pH and N.P.N. determinations. The remainder was incubated for about 4 hours at 39°C. At the end of this time a further small sample was taken for the estimation of pH and N.P.N., and the remaining volume measured and "clarified" by passing it through a Sharples Super-centrifuge. The speed of the centrifuge was approximately 24,000 r.p.m. thereby giving a force of approximately 14,500 x gravity on the surface of the rotating bowl. The liquid flowed through the centrifuge at the rate of about 3 litres per hour.

As the centrifuge slowed down, about 300 ml. of liquid, rich in smaller bacteria flowed from the rotating bowl and were collected separately. The main bulk of the bacterial sediment adhered to the sides of the bowl as a thick slime. The slime was scraped off, immediately placed in alcohol, and the mixture of sediment and alcohol ground in a mortar to produce a fine suspension of the bacteria. (Shaking was not sufficient to disperse the bacteria.) The bacterial

TABLE 10

A record of the experiments made during the preparation of a dried sample of rumen bacteria

Prep. No.	Volume incubated (litres)	pH changes			N.P.N. changes (mg./100g. rumen liquid			Yield of bacterial sediment (g.)			Total yield per litre of rumen liquid
		Initial	Final	Change	Initial	Final	Change	Main bulk	Draining bowl	Total	
1	1.50	6.9	5.5	-1.4	42.8	31.0	-11.8)				
	1.63	6.8	5.5	-1.3	42.6	31.4	-11.2)			23.4	4.8
	1.70	6.8	5.5	-1.3	41.1	29.6	-11.5)				
2	1.72	7.0	5.9	-1.1	38.8	29.1	- 9.7)				
	1.94	7.0	6.0	-1.0	36.9	26.4	-10.5)			17.1	2.9
	2.33	7.1	6.0	-1.1	34.8	24.8	-10.0)				
3	1.97	6.7	5.5	-1.2	38.3	24.0	-14.3)				
	2.20	6.6	5.3	-1.3	37.3	26.4	-10.9)			22.8	3.6
	2.10	6.5	5.3	-1.2	41.2	28.9	-12.3)				
4	1.83	7.1	5.7	-1.4	39.5	24.2	-15.3)				
	2.20	7.0	6.0	-1.0	36.6	23.2	-13.4)			20.1	3.2
	2.25	7.0	5.8	-1.2	36.1	23.2	-12.9)				
5	1.97	6.8	6.6	-0.2	31.9	26.9	- 5.0)				
	2.60	7.3	6.7	-0.6	39.5	27.0	-12.5)			15.0	3.3
6	2.10	6.2	5.2	-1.0	37.4	29.0	- 8.4)				
	2.43	6.4	5.2	-1.2	30.2	20.3	- 9.9)			20.3	3.0
	2.23	6.7	5.1	-1.6	31.5	18.6	-12.9)				
7	2.12	6.2	5.2	-1.0	27.8	17.0	-10.8)				
	2.28	6.2	5.0	-1.2	29.1	20.5	- 8.6)			22.1	5.0
8	2.18	6.9	6.4	-0.5	29.5	24.0	- 5.5)				
	2.25	7.1	6.4	-0.7	28.7	23.1	- 5.6)			11.0	2.5
9	2.43	6.6	5.1	-1.5	38.6	27.0	-11.6)				
	2.70	6.6	5.1	-1.5	37.3	26.7	-10.6)			12.4	2.4
10	2.52	6.4	5.1	-1.3	39.2	28.2	-11.0)				
	2.50	6.6	5.2	-1.4	30.2	20.2	-10.0)			12.5	2.5
11	2.45	6.6	5.4	-1.2	30.2	18.6	-11.6)				
	2.50	6.6	5.5	-1.1	27.5	16.5	-11.0)	14.8)			
								)	7.8	38.5	4.0
12	2.30	6.4	5.2	-1.2	38.6	31.1	- 7.5)	)			
	2.50	6.4	5.3	-1.1	41.7	34.8	- 6.9)	15.9)			
13	2.40	6.7	-	-	-	-	-)				
	2.32	6.7	5.3	-1.4	34.1	22.7	-11.4)	16.2	3.6	19.8	4.2
14	2.10	6.6	5.2	-1.4	32.6	23.0	-12.6)				
	1.95	6.7	5.5	-1.2	35.5	27.5	- 8.0)	15.7	3.3	19.0	4.7
15	2.30	6.5	5.3	-1.2	31.8	21.3	-10.5)				
	2.48	6.5	5.3	-1.2	28.7	18.8	- 9.9)	16.1	3.6	19.7	4.1
16	2.33	6.8	5.3	-1.5	31.7	20.6	-11.1)				
	2.45	6.8	5.4	-1.4	29.3	20.2	- 9.1)	13.2	3.8	17.0	3.6
17	2.40	6.4	5.1	-1.3	35.3	27.7	- 7.6)				
	2.56	6.5	5.2	-1.3	35.2	27.8	- 7.4)	14.1	3.8	17.8	3.6
18	2.35	6.5	5.4	-1.1	38.0	26.9	-11.1)				
	2.40	6.5	5.4	-1.1	28.1	20.4	- 7.7)	15.2	3.4	18.6	3.9
19	2.40	6.5	5.3	-1.2	34.7	26.7	- 8.0)				
	2.45	6.5	5.4	-1.1	36.5	27.6	- 8.9)	15.4	4.6	20.0	4.1
20	2.47	6.5	5.5	-1.0	31.9	22.9	- 9.0)				
	2.47	6.6	5.7	-0.9	32.1	23.4	- 8.7)	13.1	3.5	16.5	3.3
21	2.33	6.6	5.6	-1.0	36.6	27.2	- 9.4)				
	2.52	6.7	5.8	-0.9	35.5	27.6	- 7.9)	12.9	3.4	16.3	3.4
22	2.30	6.4	5.7	-0.7	32.8	26.7	- 6.1)				
	2.54	6.6	6.0	-0.6	26.6	20.5	- 6.1)	11.8	2.3	14.1	2.9
23	1.91	6.5	5.4	-1.1	36.9	26.3	-10.6)				
	2.28	6.6	5.6	-1.0	31.8	23.4	- 8.4)	11.6	3.0	14.6	3.5
24	2.12	6.1	5.1	-1.0	39.3	30.4	- 8.9)				
	2.75	6.3	5.3	-1.0	35.7	27.4	- 8.3)	13.2	2.1	15.3	3.1
25	2.30	6.5	5.2	-1.3	37.2	27.5	- 9.7)				
	2.56	6.6	5.4	-1.2	37.9	28.7	- 9.2)			17.5	3.6
26	2.52	6.5	5.4	-1.1	38.1	28.8	- 9.3)				
	2.56	6.4	5.5	-0.9	38.4	30.3	- 8.1)	16.2	3.6	19.8	3.9

Total 129.95 litres

Total = 461.2 g.

∴ Average yield = 3.55 g. per litre

suspension was then centrifuged and washed twice with alcohol and twice with ether, centrifuging between each washing. The residue was transferred to weighed petri dishes and the solvents allowed to evaporate, leaving a fine greyish-white powder, which when examined under the microscope was found to consist mainly of bacteria and bacterial debris. The weight of the powder was recorded.

The liquid which drained from the bowl of the Sharples centrifuge as it came to a standstill, was poured into alcohol and treated with alcohol and ether as already described for the main portion of the bacteria. The resulting powder is referred to in the present description of the work as "draining bowl sediment".

A record of the whole series of experiments is shown in Table 10. In the earlier batches the draining bowl sediment was not recovered separately, but since it was finally mixed with the bulk after chemical and microscopical examination, the experiments were comparable throughout. The dry preparation from each experiment was stored in a refrigerator until the final batch was prepared. The bulked preparation was then finely ground, passed through a 90 gauge wire sieve and thoroughly mixed.

Analysis of the dried bacteria Before all the batches of dried bacteria were mixed, a random selection were analysed for moisture, protein and polysaccharide. Samples of the product obtained from the draining bowl

liquid were also analysed separately. The latter were found to be slightly richer in protein and poorer in polysaccharide than the main preparations. Samples of the main material and of the draining bowl material were examined by Mr. Frank Baker who has done so much work on the microbiology of the rumen (e.g. Baker, 1943; Smith and Baker, 1944). He reported that the main fraction had a much higher count of macro- and micro-iodophiles than the draining bowl fraction, the latter consisting mainly of "minute aniodophile or non-reactant iodophile cocci and diplococci". Since the poly-saccharide content of the aniodophile bacteria tends to be low this report agrees with the finding that the polysaccharide content of the draining bowl fraction was lower than that of the other.

Since the object was to determine the nutritive value of the protein of the mixed flora rather than of any fraction of it, all the batches were mixed, ground up, seived and analysed. The methods used for the analysis have been described already on pp. 24-27. The results of the analysis are recorded in Table 11, where they are compared with typical figures for dried fodder yeast and for a typical concentrate feedingstuff, linseed cake. It will be observed that the protein and ash contents of the dried rumen preparation lay between those of the linseed cake and the dried yeast, while the carbohydrate content was of the same order for all three.

The presence of so much phosphorus and pentose

TABLE 11

The composition of the bulk sample of dried rumen bacteria compared with published values for dried fodder yeast and for linseed cake, a typical concentrate feedingstuff

	Composition of dried rumen bacteria	Dried rumen bacteria	Fodder Yeast (Braude, 1942)	Linseed cake
		Composition on dry weight basis		
	%	%	%	%
Moisture	7.5	0.0	0.0	0.0
Crude protein (Nx6.25)	41.1	44.4	52.6	33.2
Total carbohydrate other than fibre	37.3	40.3	36.1	40.0
Lipoid matter	2.8	3.1	1.1	10.7
"Crude fibre"	0.3	0.3	0.9	10.2
Ash	6.6	7.1	9.3	5.9
Total	95.6	95.2	100.0	100.0

Note: The sample of dried bacteria having 7.5% moisture containing 4.1% pentose which is included in the carbohydrate figure of 37.3%. It also contained 1.25% phosphorus and 0.27% calcium.

suggested that the preparation contained a fair proportion of nucleoprotein. Since it has been stated that the ordinary Kjeldahl procedure does not always convert purine nitrogen to ammonia quantitatively, some doubt existed as to whether the total nitrogen was being estimated. To ensure that purine rings are broken down completely it is usual to heat the substance with a strong oxidising agent such as "Perhydrol" in presence of sulphuric acid. This was done, but the value obtained for the nitrogen was the same as by the ordinary Kjeldahl process.

The high ash and phosphorus content of the bacterial preparation is typical of that found for yeasts (Table 11) and other organisms. For example Guilleman and Larson (1922) found 5.45% ash from the fixed salts of B. Coli and of this 1.85% was due to P_2O_5 not in combination with calcium. In the rumen preparation there was 7.10% ash, and of this 1.35% was due to phosphorus and 0.29% to calcium (Table 11). It can be calculated, therefore, that the phosphorus present as $Ca_3(PO_4)_2$ amounted to 0.15% which leaves 1.2% to be distributed between other substances such as phosphatides, protein and nucleic acids. Assuming that the pentose figure of 4.4% represents ribose incorporated in tetra-nucleotides it can be calculated that the product may have contained as much as 9.45% nucleic acid, which seems very high. Boivin, Vendrely and Tulasne (1947), however, in a review of the subject concluded that the nucleic acid content of many

different bacteria varied from 5 to 16% of their dry weight. Assuming that there was 9.45% nucleic acid, this would contain 0.9% phosphorus and leave 0.3% distributed between substances such as protein and phosphatides. Work on the nature of the nucleic acids of rumen bacteria is at present being planned.

It will be seen from Table 11 that the amount of the dried preparation accounted for was 95.6%. Repeated attempts have been made to identify the remaining 4.4% but so far without success.

The proportion of the preparation synthesised during incubation The preliminary tests mentioned on p. 50 indicated that the amount of protein synthesised during incubation might represent about 34% of the total protein in the preparation. This figure was only for one test and it undoubtedly varied with different samples of rumen liquid. An estimate of the amount of protein in the final pooled sample which was synthesised during the various incubations can be gained from the data in Table 10. In the fifty-seven incubations for which the results are recorded, the total amount of N.P.N. converted to protein nitrogen was 12.6 g. representing 78.8 g. protein. The total weight of the dried preparation was 461 g. with a protein content of 41.1%, the total amount of crude protein in the preparation being 189.4 g. It follows, therefore, that about 58% of the bacterial protein was present originally when the rumen liquid was taken from the

rumen, and that about 42% was synthesised during incubation.

The purity of the preparation Before experiments were carried out to determine the nutritive value of the dried bacterial preparation, it was important to know whether the material was contaminated to any significant degree with feedingstuffs or feedingstuffs protein from the rumen. The fact that the preparation consisted mainly of bacteria and bacterial debris and that any contamination which may have existed was very slight, was indicated by microscopical examination and also by the following considerations.

(a) The "fibre" content of the preparation was estimated by the method recommended by the Association of Official Agricultural Chemists (1945) as outlined on p.26 and was found to be 0.3%. Confirmation of this figure was sought by the newer method of Whitehouse, Zarow and Shay (1945) which is briefly described on p.26. At the end of digestion by this latter method there was no detectable amount of "fibre" on the filter cloth, and this would normally lead to the conclusion that no fibre was present in the sample being analysed. The filtrate, however, was cloudy and on centrifuging it, some solid material was obtained which on incineration gave a loss in weight equivalent to 2.1% of the original sample. It was possible, therefore, that if any fibre was present it was so finely divided that it passed through the filter cloth. To test whether this was

likely to be so, the following procedure was adopted. Some of the sludge which was obtained by centrifuging fresh rumen contents for 5 minutes at 2,000 r.p.m. and which would certainly contain vegetable matter rich in fibre was washed with alcohol and ether and dried. The dry product was ground in a mortar and passed through the same sieve which was used in preparing the dried rumen bacteria. The sieved material was then analysed for fibre and was found to contain 10.5% of unhydrolysable material which was retained on the filter cloth. It is probable, therefore, that had the dried rumen bacteria contained measurable amounts of fibre derived from the feedingstuffs, this fibre would have been retained on the filter cloth, and that the 2.1% of combustible material which passed through the cloth but was recovered from the filtrate when the batterial preparation was analysed, was not fibre.

It is interesting to note from the analytical figures in Table 11 that dried yeast may contain as much as 0.9% "fibre", and since this could not possibly arise from contamination with ordinary vegetable matter it lends support to the conclusion that the 0.3% "fibre" found by the official method in the rumen bacterial preparation in the present work was not necessarily of dietary origin. Furthermore, it should be noted that when Johnson et al. (1944) made a preparation of rumen bacteria by growing certain strains on synthetic medium from which feedingstuffs were absent, the product contained 0.25% "fibre", whereas the preparation which

TABLE 12

The amount of N.P.N. and protein nitrogen extracted by water and by rumen contents from feedingstuffs under various conditions

Expt.	Conditions of extraction	Feedingstuff added	N extracted in mg./100g. extracting liquid		
			N.P.N.	Protein N	∴ total N
1	10g. feedingstuff extracted with 200g. water at 37°C for 2½ hr.	Bean meal	30.0	73.5	103.5
		Bruised oats	13.1	5.7	18.8
		Dried grass	17.3	2.7	20.0
2	Rumen contents alone	Control	23.0	39.5	62.5
	12.5g. feedingstuff stirred with 250g. of rumen contents at 37°C for ¼ hr.	Bean meal	46.6	44.3	90.9
		Mixture of dried grass, bean meal, bruised oats	35.7	37.8	73.5
3	Rumen contents alone	Control	30.1	34.9	65.0
	5g. feedingstuff stilled with 250g. rumen contents at 37°C for 1 hr.	Mixture of dried grass, bean meal, bruised oats	34.8	32.2	67.0
		Mixture of concentrate cubes, bruised oats, dried grass, bean meal	36.4	33.3	69.7

the same authors made direct from rumen contents contained 1.36%, a figure which may well indicate an appreciable degree of contamination.

From the fibre content of the various constituents of the rations which are listed on p. 50 it can be calculated that the overall fibre content of the diet which was fed in the present work was about 14%. The value of 0.3% found by the official method for the dried rumen preparation indicates, therefore, that contamination with feedingstuffs was very slight - probably less than 2 or 3%.

(b) The possibility that the bacterial preparation might contain protein derived direct from feedingstuffs without being contaminated with fibre was also investigated. An experiment was first made in which various feedingstuffs were extracted with water at 37°C for 2½ hours. The feedingstuffs were removed by the centrifuging procedure normally employed in this work to remove vegetable matter from rumen liquid prior to incubation, and the amount of N.P.N. and protein nitrogen remaining in the supernatant layer estimated. From the results given in Table 12, it was concluded that beans were the most likely constituent of the rations to cause serious contamination. The bean extract was clarified in the Sharples centrifuge and the sediment washed with alcohol and ether and dried in the usual way. A white powder was obtained which was found to consist of 97.3% protein, and to contain no appreciable amount of carbohydrate or pentose. Furthermore this protein

material was shown to be almost wholly soluble in 5% saline, which makes it unlikely that it would be centrifuged out of rumen liquid during the preparation of the dried bacteria. However, to test this possibility ground beans were mixed with unstrained semi-solid rumen ingesta to imitate the conditions in the rumen and vigorously stirred for 15 minutes. The mixture was then strained through muslin and centrifuged in the usual way. A second aliquot of ingesta without addition of bean meal was treated similarly as a control. The resulting liquid was then analysed for N.P.N, and protein. Once more it was shown that N.P.N. and protein nitrogen had been extracted from the beans, but the amount of protein nitrogen extracted was much less than it had been with water (Experiments 1 and 2, Table 12). The same test was made using a mixture consisting of equal parts of bean meal, bruised oats and dried grass, instead of bean meal alone, and under these conditions no protein nitrogen appeared to be extracted from the feedingstuffs. In fact the value for the protein nitrogen was only 37.8 mg. per 100 g. of extracting liquid as compared with 39.5 in the control. In a third series of experiments carried out to explore the matter still further, bean meal was mixed with equal parts of dried grass and bruised oats, all constituents of the diet when the rumen bacteria were isolated, and these three constituents with and without typical concentrate feedingstuff cubes were incubated with rumen ingesta for an hour instead of simply being stirred with

the ingesta for 15 minutes (Experiment 3, Table 12). Again in this experiment no protein appeared to be extracted from the feedingstuffs by the rumen liquid.

These considerations confirm that any significant amount of contamination with protein derived from feedingstuffs was most unlikely and that the dried preparation consisted almost entirely of bacteria and bacterial debris.

The nutritive value of the protein of the bacterial preparation Owing to the fact that small experimental animals were not then available at the Hannah Institute, the bacterial preparation was sent to Dr. S.K. Kon and Dr. K.M. Henry, the National Institute for Research in Dairying at Reading, who kindly undertook to test its nutritive value by feeding experiments with rats. The method used was essentially that of Mitchell (1924,1) and Mitchell and Carman (1926), the details of which have been fully described by Henry, Kon and Watson (1937). The bacterial preparation was fed to 12 female rats in a diet of which it formed 18.1%, the remaining constituents being cane sugar 15.0%, potato starch 10.0%, rice starch 42.9%, margarine fat 10.0% and salts 4.0%. The diet contained 8% protein all of which was supplied by the bacterial preparation. Metabolic faecal nitrogen and endogenous urinary nitrogen were estimated during periods of 6 days before and after the 6 day experimental period. The data supplied by Dr.Henry and Dr.Kon and the biological values and digestibilities calculated from

TABLE 13

The results of biological value and digestibility tests carried out by Dr. K.M. Henry and Dr. S.K. Kon with the dried preparation of rumen bacteria

Rat No.	Initial low egg-nitrogen period					Experimental period								Final low egg-nitrogen period				
	Weight of rat (g.)		Diet intake	Faecal N	Urinary N	Weight of rat (g.)		Diet intake	N intake	Faecal N	Urinary N	Biological value	True digestibility	Weight of rat (g.)		Diet intake	Faecal N	Urinary N
	Initial	Final	(g.)	(mg.)	(mg.)	Initial	Final	(g.)	(mg.)	(mg.)	(mg.)			Initial	Final	(g.)	(mg.)	(mg.)
1	54	56	34.72	85.3	70.9	58	70	47.49	617.0	262.0	120.8	88.9	74.7	74	80	46.10	91.9	68.7
2	55	59	40.71	91.6	76.0	62	69	41.64	541.1	226.7	117.7	90.3	75.2	70	76	42.13	92.4	77.4
3	58	60	39.29	88.2	78.9	64	74	49.48	642.8	274.7	139.6	87.6	73.7	78	89	55.18	112.0	84.1
4	56	58	33.60	72.5	75.3	60	68	48.24	626.7	270.1	144.4	86.0	73.8	70	77	44.04	98.0	86.4
5	56	62	47.59	104.0	78.2	65	72	52.49	681.9	296.7	145.2	88.2	72.4	77	87	57.11	111.3	99.9
6	55	62	44.48	88.4	76.1	66	76	52.49	681.9	288.8	130.3	90.0	72.7	80	89	58.35	112.8	82.6
7	56	59	37.45	79.7	75.8	63	70	51.47	668.8	305.2	178.8	81.1	71.4	72	75	44.43	101.3	99.0
8	49	56	48.63	97.9	76.2	60	67	51.98	675.4	319.3	149.2	88.1	69.2	70	72	40.96	92.9	106.3
9	46	51	42.81	74.9	79.9	55	66	51.98	675.4	273.2	131.7	90.5	74.7	70	79	58.70	128.2	84.2
10	48	53	38.77	81.1	73.0	56	68	51.99	675.6	280.6	130.5	89.8	74.3	73	78	48.99	98.7	83.7
11	54	54	25.88	57.8	78.1	56	66	45.35	589.2	257.1	126.0	87.2	72.6	72	80	49.24	97.4	67.7
12	54	58	42.89	91.0	69.9	63	73	52.48	681.9	293.7	121.7	91.1	73.9	78	82	51.85	119.0	80.5
											Mean	88.2	73.2					
											S.E.M.	±0.79	±0.56					

the data are shown in Table 13. The mean of the biological values for the 12 individual rats was 88.2 and of the true digestibility values it was 73.2.

Discussion

The value of 88.2 for the biological value of the bacterial protein is high and compares favourably with values found by the same technique for first class protein of animal origin fed at a similar level. It is considerably higher than that of 66 found by Johnson *et al.* (1944) for their preparation, but it must be noted that the American workers had only sufficient material to feed to three rats and that one of the three individual values obtained by them was 56 while the others were 70 and 73. Moreover, the digestibility of their preparation was only 54.9% and this, considered together with the fibre content of 1.36%, (which has already been referred to on p.58), suggests that their preparation may have been seriously contaminated with products derived from feedingstuffs, and so the low biological value which they obtained may have been due to contamination with vegetable protein.

The value of 73.2 found for the digestibility of the bacterial protein in the present work is low compared with the values usually obtained for common dietary proteins in similar experiments with rats, but it compares well with the figure of 74 obtained some years ago by Mitchell (1924,2) for the digestibility of the nitrogen of

yeast, and it may be that in the normal rumen the digestibility of part of the bacterial protein is increased by being ingested by protozoa before it is digested by the host animal. Whether this occurs or not, the fact remains that the biological value of 88 and the digestibility of 73 found in the present work indicate a protein of relatively high nutritive value.

A Note on the Rumen Protozoa

Recent work by Pearson and Smith (1943, 3) and Smith and Baker (1944) showed that the protein synthesis which occurs during *in vitro* incubations does not require the presence of the rumen protozoa. It is possible, however, that although the protozoa play no important part in the conversion of N.P.N. to protein, they may render the bacterial protein more readily available to the host animal by ingesting the protein and building it into their own protoplasm. Certainly it would appear that a portion of the *dietary* protein may be utilized and changed by the protozoa for it has been claimed that their numbers vary regularly with the protein content of the diet (Mowry and Becker, 1930; Van der Wath and Myburgh, 1941). To assess the importance of the protozoa in the protein metabolism of the rumen it will be essential to know the biological value of their protein. Johnson *et al.*(1944) obtained biological values of 60 and 75 but their experiments involved only 2 rats and it is clear that more experiments on a larger scale are required. Plans have, therefore, been

drawn up for preparing sufficient of a sample of dried protozoa to enable a thorough test to be made, but this section of the work is not yet sufficiently advanced to describe here.

To prepare the dried protozoa the rumen liquid is strained through muslin and centrifuged for 5 minutes at 2,000 r.p.m. (centrifugal force, 800 x gravity). The sludge so obtained is suspended in water and allowed to sediment in a 2 litre cylinder. The supernatant layer is then syphoned off and the white layer of protozoa suspended again in water and the process repeated several times in an attempt to remove vegetable matter. The product is then washed twice with alcohol and twice with ether and dried. So far, analysis of the dry material shows it to contain 38% protein, 44% hydrolysable polysaccharide, 8% lipoid matter and 9% "fibre". It is of interest to note that polysaccharide is present in the protozoa in the rumen to much the same extent as it is present in the bacteria after incubation in the presence of sugar (Table 11). The value of 9% for the "fibre" content may be due in part to contamination with feedingstuffs but it may also result partly from the fact that the protozoa are known to ingest small vegetable particles which can commonly be seen inside the organisms when examined microscopically (see Baker, 1943).

Conclusions and Summary

About 460 g. of dried rumen bacteria have been prepared. Some 42% of the protein of this preparation was synthesised from N.P.N. during *in vitro* incubation, the remainder being present in the bacteria when they were removed from the rumen. Microscopical and chemical examination of the material suggested that there was very little contamination from feedingstuffs. The similarity in composition between this preparation and a preparation of food yeast has been noted.

The results of biological value and digestibility trials with rats receiving a diet containing 8% of protein supplied by the bacterial preparation showed that the biological value of the bacterial protein was 88.2% and the digestibility 73.2%. From this it is concluded that microbial protein passing from the rumen to the other stomachs and small intestine of the ruminant becomes available to the host animal and that it is likely to be of high biological value to the host.

Further work is required to determine the amount of bacterial protein which becomes available to the animal in a given time, and to determine more exactly the conditions *in vivo* which encourage the maximum use of N.P.N. for protein synthesis by the bacteria.

Work similar to that described for the rumen bacteria has been begun with rumen protozoa.

Part III. The Effect of Animal Management Factors on the synthesising Power of Rumen Liquid in vitro

Introduction

In the earlier work of Pearson and Smith (1943, 3), and also in the present work, it was observed that when samples of rumen liquid were incubated for a period of 4 hours under strictly controlled conditions, the amount of protein synthesis which occurred per 100 g. rumen liquid varied greatly from sample to sample. Thus on one day there might be a synthesis equivalent to 10 mg. N per 100 g. rumen liquid, whereas on another day the synthesis might be equivalent to only 2 mg. N/100 g. Differences in "synthesising power", whatever their cause, may be of great importance in the utilization of N.P.N. by the ruminant, and certainly a brief glance at the literature dealing with the subject of N.P.N. utilization confirms the view that the amount of N.P.N. which appears to be converted to protein can vary greatly from one animal to another and from one set of experiments to another. McAnally (1943) working with rumen contents in vitro noted that the rate of fermentation depended on three factors, namely the type of carbohydrate being fermented, the concentration of the carbohydrate and the nature and time of withdrawal of the rumen contents from the rumen. The first two factors can be controlled in the laboratory, but the

third is connected with the management of the fistula animals. Furthermore, Elsden (1945-46) when studying the fermentation of glucose *in vivo* showed that when poor quality hay was being fed and the animal was in relatively poor condition, glucose introduced into the rumen was fermented very slowly, but substitution of good quality clover hay for that previously fed resulted in rapid fermentation of glucose similarly administered.

The work described in the present and following sections (Parts 3 and 4) was planned with the object of determining sone of the factors which affect the "synthesising power" of the rumen liquid, the present section being devoted mainly to what may be termed animal management factors.

Experimental

1. *The position in the rumen from which the sample is taken* In earlier work Pearson and Smith (1943, 1) found great difficulty in obtaining representative samples of the material in the rumen. The composition differed considerably when samples were taken from different positions in the paunch. It was for this reason that the present technique was evolved of taking as liquid a sample as possible and straining it through muslin before using it for *in vitro* incubations. Before carrying out the following experiments, it was felt desirable to determine whether the nitrogen partition in the strained liquid varied appreciably according to the position from which the sample was taken. The four

TABLE 14

The analysis of four samples of rumen liquid all obtained at one time from four different positions in the rumen

The position from which the sample was taken	pH	Total N	N.P.N.	Protein N by difference	NH_3-N
	(Mg. N/100g. rumen liquid)				
1. Half-way down into the rumen at the eosophageal end	7.10	73.8	20.6	53.2	14.5
2. Half-way down into the rumen at the end furthest from the eosophagus	7.19	76.8	21.8	55.0	15.0
3. Deep in the rumen	7.12	81.6	20.4	61.2	15.1
4. Near the surface of the rumen contents	7.01	102.5	23.2	79.3	16.6

samples of liquid detailed in Table 14 were removed from four different positions in the rumen and their content of total nitrogen, N.P.N. and ammonia-nitrogen determined. The results recorded in Table 14 show that the sample taken from near the surface had a higher protein content than the others but that the N.P.N. and ammonia contents were much the same for all the samples, and since protein synthesis seems to involve the ammonia only (Pearson and Smith, 1943, 3) it is most unlikely that it matters from what part of the rumen the liquid is taken.

2. The time at which the sample is taken The course of fermentation in the rumen as it changes throughout the day has been followed by various workers. The decrease in pH and the formation of volatile fatty acids are greatest in the few hours following a meal, when fermentation appears to be at its peak, the exact time depending on the nature of the feed. (Monroe and Perkins, 1939; Hale, Duncan and Huffman, 1940; Phillipson, 1942). In this connection also, Johnson et al. (1944), who made counts of the rumen fauna and flora during the 24 hours following a meal, demonstrated that the greatest number of bacteria occurred approximately one hour after feeding. Experiments were carried out, therefore, in the present work to determine whether the synthesising power of the rumen liquid was also greatest one hour after feeding.

Samples of rumen contents were removed just prior to feeding and then one hour and approximately

TABLE 15

The synthesis obtained with samples of rumen liquid removed from the rumen before and after feeding

Experiment No.	Time of sampling	No urea added						Urea added		
		pH			N.P.N.			N.P.N.		
					(a)	(b)	(a-b)	(c)	(d)	(c-d)
					(mg.N/100 g. rumen liquid)					
		Initial	Final	Change	Initial	Final	Synthesis	Initial	Final	Synthesis
1	Fasting	6.74	6.03	-0.71	9.5	6.3	3.2	31.9	27.4	4.5
	1 hr.after feeding	6.50	5.61	-0.89	14.1	11.4	2.7	35.8	29.8	6.0
	4½ hr. " "	6.44	5.68	-0.76	9.6	8.1	1.5	30.9	27.9	3.0
2	Fasting	6.94	6.68	-0.26	12.8	11.6	1.2	35.6	32.7	2.9
	1 hr.after feeding	6.89	6.06	-0.83	25.5	21.5	4.0	47.6	42.3	5.3
	4½ hr. " "	6.71	6.07	-0.64	15.0	12.1	2.9	37.6	32.9	4.7
3	Fasting	6.42	6.14	-0.28	4.5	2.9	1.6	24.5	18.7	5.8
	1 hr.after feeding	6.24	5.53	-0.71	8.7	4.2	4.5	32.5	21.3	11.2
	4½ hr. " "	6.42	6.19	-0.23	3.3	2.6	0.7	26.7	21.7	5.0
4	Fasting	6.58	6.50	-0.08	3.6	3.4	0.2	25.6	22.3	3.3
	1 hr.after feeding	6.34	6.05	-0.29	6.8	4.2	2.6	21.0	13.5	7.5
	4½ hr. " "	6.36	6.55	+0.19	3.6	4.1	-0.5 (hydrolysis)	25.2	25.3	-0.1 (hydrolysis)
5	Fasting	6.64	6.20	-0.44	4.0	2.3	1.7	24.8	20.5	4.3
	1 hr.after feeding (concentrates contained urea	6.44	5.55	-0.89	20.9	14.1	6.8	45.7	33.7	12.0
	4½ hr.after feeding	6.25	5.92	-0.33	4.5	3.3	1.2	25.9	20.1	5.8

4½ hours after food had been ingested. The material was strained through muslin and centrifuged as described on p.18. Maltose (1%) was added to each sample of rumen liquid and it was then divided into two portions. One portion was incubated without added urea and the other with the addition of about 50 mg. urea per 100 g. rumen liquid. The rumen contents used for Experiments 1 and 2 were obtained from a bullock with a fistula, whereas the material used for Experiments 3, 4 and 5 was obtained the following year from a cow with a fistula. The results of these five experiments are recorded in Table 15.

The effect of adding urea will be discussed later. The main points to be observed from Table 15 at this stage, are that the amount of synthesis obtained in all but the first experiment was very much greater one hour after a meal than it was either just before the meal or 4½ hours after it, and that for all five experiments the pH change occurring on incubation suggested that the formation of volatile fatty acids was also greatest for the sample taken one hour after a meal. In general, therefore, it may be concluded that the activity of the rumen liquid, including its "synthesising power", is greatest just after a meal. This agrees with the finding of Johnson et al. (1944) that the number of bacteria in the rumen contents is also greatest at that time.

3. The effect of the nature of the diet on the levels

TABLE 16

The effect of adding N.P.N. to the rumen liquid on the amount of synthesis obtained

Experiment No.	Amount of N.P.N. added	Initial N.P.N.	Final N.P.N.	Synthesis
		(a)	(b)	(a - b)
	mg. N / 100 g. Rumen liquid			
1	0.0	11.5	11.0	0.5
	8.0	19.5	17.3	2.2
	18.8	30.3	22.7	7.6
	51.0	62.5	55.0	7.5
2	0.0	5.2	4.5	0.7
	20.3	25.5	24.1	1.4
	27.6	32.8	30.1	2.7
	48.0	53.2	52.0	1.2
3	0.0	16.8	16.1	0.7
	3.9	20.7	19.8	0.9
	9.4	26.2	24.4	1.8
	19.7	36.5	31.9	4.6

of N.P.N. and protein in the rumen liquid and on its synthesising power It was thought possible that the synthesising power of a sample of rumen liquid might be related to the level of protein, N.P.N. or ammonia which it contained, and that this in turn might depend on the nature of the diet of the host animal. A series of experiments was planned, therefore, with the object of collecting information on this aspect of the subject.

In a preliminary test, a sample of rumen liquid of relatively low N.P.N. content and containing 1% of added maltose, was divided into four portions. One of the portions was incubated without any further addition, while the others were incubated after different amounts of urea had been added. The results recorded in Table 16 for the first experiment show that for this particular sample the synthesis was negligible when no N.P.N. was added, but that when the N.P.N. level was increased from 11.5 to 30.3 mg./100 g. rumen liquid, an amount of synthesis occurred which was equivalent to 7.6 mg. nitrogen per 100 g. rumen liquid. On raising the N.P.N. level further, however, to 62.5 mg./100 g. there was no further increase in the amount of synthesis. As would be expected, therefore, there is a limit to the amount by which the synthesis can be enhanced. This general increase in the amount of synthesis on raising the N.P.N. level was also observed in two other experiments of a similar type for which the results are also recorded in Table 16.

TABLE 17

The nitrogen partition in the liquid obtained from the rumen when a number of different diets were being fed

(The rumen liquid was strained through muslin but not centrifuged)

Rations per day	(n) = Number of samples (M) = Mean (S.E.M.) = Standard error of mean	pH	Total N	N.P.N.	NH_3-N
			mg. N/ 100 g. rumen liquid		
A. Hay..... 10 lb. Turnips .... 7 " *Concentrates 4 "	n M S.E.M.	22 6.45 ±0.065	22 128.4 ±9.274	22 17.8 ±1.21	22 12.8 ±0.96
*Equal parts of bruised oats, bean meal and dried grass.					
B. Hay..... 10 lb. *Concentrates 4 "	n M S.E.M.	11 6.30 ±0.062	11 96.1 ±8.27	11 14.0 ±1.08	11 9.8 ±0.95
*Bean meal 3 parts, dried grass 2 parts, vetch meal 1 part, bruised oats 1 part					
C. Hay..... 14 lb. *Concentrates 6 "	n M S.E.M.	14 6.42 ±0.057	14 90.4 ±8.04	14 11.7 ±0.48	14 8.4 ±0.49
*Barley 2 parts, bean meal 1 part, dried grass 1 part.					
D. Hay..... 16 lb. Cabbage..... 20 " *Concentrates 6 "	n M S.E.M.	9 6.70 ±0.053	9 96.7 ±4.14	9 12.1 ±0.92	9 8.9 ±0.89
*Bruised oats 3 parts, dried grass 3½ parts, bean meal 1½ parts.					
E. Hay..... 10 lb. *Concentrates 4 "	n M S.E.M.	5 6.20 ±0.064	5 117.8 ±14.95	5 18.8 ±1.81	5 13.1 ±1.76
*Equal parts of bruised oats, bean meal and dried grass.					
F. Hay..... 10 lb. *Concentrates 4 "	n M S.E.M.	9 6.39 ±0.039	9 66.1 ±4.53	9 13.0 ±1.87	9 10.9 ±1.13
*Equal parts of bruised oats and dried grass, mixed with urea (3%) dissolved in molasses.					
G. Hay..... 12 lb. *Concentrates 8 "	n M S.E.M.	4 6.50 ±0.108	4 92.6 ±3.17	4 8.6 ±1.16	2 6.9 ±1.81
*Equal parts of bruised oats, bean meal and dried grass.					
H. Hay..... 10 lb. *Concentrates 4 "	n M S.E.M.	2 6.09 ±0.310	2 96.0 ±15.20	2 8.0 ±1.96	2 4.6 ±1.80
*Bruised oats 13 parts, bean meal 2 parts, dried grass 1 part.					
J. Pasture grass	n M S.E.M.	8 6.64 ±0.068	8 152.2 ±9.55	12 29.1 ±2.06	7 21.2 ±2.22

In view of this relationship which appeared to exist between the level of N.P.N. and the amount of synthesis produced, it was decided to keep the fistula animals on a series of different diets for periods of several weeks and determine whether the level of N.P.N. and ammonia in the rumen liquid varied with different diets and whether the synthesising power of the liquid was related to any variations which might be observed. With this object in view the nitrogen partition was determined in a number of samples taken from the rumen when different diets were being fed. The pH of the rumen contents on their removal from the rumen was also noted. Except when the animals were grazing the samples were taken from the rumen about 1½ hours after the animals had been fed. Each diet was fed for some days before sampling was begun in order to ensure that the conditions in the rumen corresponded to that particular diet.

Means and the standard error of the means have been calculated for the numerous results which were obtained, and are shown in Table 17. The following general observations were made. 1. For any one type of diet, the N.P.N. level in the rumen liquid varied considerably from day to day. Thus on diet A, for example, the values ranged from 6.4 to 25.8 mg./100 g. rumen liquid. 2. The N.P.N. level when the animals were receiving grass was generally much higher than when the animals were stall-fed. This tendency has been noted many times in the course of the present work.

TABLE 18

The nitrogen partition in centrifuged rumen liquid when a number of different diets were being fed

(Before analysis the rumen liquid was centrifuged to remove protozoa and most of the dietary vegetable matter)

Ration *	No. of samples analysed		Composition of centrifuged rumen liquid (mg.N/100g. rumen liquid)		
			Total N (a)	N.P.N. (b)	Protein N (a-b)
A	3	Mean	27.9	17.1	10.8
		Standard error of mean	± 2.81	± 1.97	± 1.16
B	12	Mean	32.0	12.3	19.6
		S.E.M.	± 1.17	± 0.73	± 0.73
E	8	Mean	27.8	12.8	15.0
		S.E.M.	± 2.88	± 2.00	± 1.46
F	12	Mean	25.9	11.1	14.8
		S.E.M.	± 2.13	± 1.66	± 1.04
H	2	Mean	21.6	5.4	16.2
		S.E.M.	± 2.00	± 0.20	± 1.80
J	5	Mean	60.7	20.3	40.4
		S.E.M.	± 3.70	± 2.45	± 5.19

* See Table 17.

3. The inclusion of turnips in the rations (diet A) may have tended to increase the N.P.N. level slightly.
4. On all the diets, from 43.4 to 95.6% of the N.P.N. consisted of ammonia, the average being 72.9%.
5. There appeared to be no relationship between N.P.N. level and the pH of the rumen liquid.

With all the diets, the figures for the total nitrogen of the rumen liquid varied greatly from sample to sample, but this would be expected since it has been found that even after straining samples through muslin, the proportion of liquid to finely divided solid matter present in the samples varies very considerably.

The results which have just been discussed were obtained by analysing rumen liquid which had been strained through muslin to remove larger vegetable particles, but which had not been centrifuged, and it was thought possible that a relationship might be found to exist between the nature of the diet and the protein level in a sample of rumen liquid, provided the liquid were first centrifuged for 5 minutes at 2,000 r.p.m. (centrifugal force, 800 x gravity) to remove the protozoa and most of the dietary vegetable matter. Portions of a number of the samples which were used for obtaining the data in Table 17 were therefore centrifuged and the total and non-protein nitrogen in the centrifuged liquid determined. The results summarised in Table 18 led to the following conclusions. 1. As with the uncentrifuged liquid, the highest N.P.N. values were obtained when the animals were grazing (Diet J).

TABLE 19

The synthesis obtained with and without added urea in samples of rumen liquid having different levels of N.P.N. and obtained from the rumen when the fistula animals were receiving different diets

Ration **		Protein and N.P.N. content of the rumen liquid (mg.N/100 g. rumen liquid)		Amount of synthesis (mg.N/100 g. rumen liquid)	
		Protein nitrogen	N.P.N.	No added urea	Urea added
A		10.9	20.4	5.0	9.0
		8.7	13.6	5.6	6.1*
	Mean	9.8	17.0	5.3	7.6
	S.E.M.	±1.1	±3.40	±0.30	±1.45
B		16.9	13.3	2.9	8.4
		19.5	16.6	2.0	6.0
		20.5	11.5	5.0	5.1*
		20.6	10.8	3.3	8.6
		20.4	8.5	4.2	7.5
	Mean	19.6	12.1	3.5	7.1
	S.E.M.	±0.49	±1.35	±0.52	±0.68
E		11.6	14.8	0.8	3.6
		16.3	16.5	0.7	4.7
		16.1	15.1	5.5	7.9
		21.9	8.7	4.5	11.2
		12.7	6.0	1.6	4.7
		8.9	6.8	2.7	7.5
	Mean	14.6	11.3	2.6	6.6
	S.E.M.	±1.86	±1.89	±0.81	±1.15
F		14.5	13.5	2.9	4.6
		17.7	6.4	5.2	5.9*
		17.6	8.5	3.8	9.8
		11.4	19.0	7.0	7.8*
		21.2	20.9	6.9	12.0
	Mean	16.5	13.7	5.2	8.0
	S.E.M.	±1.66	±2.83	±0.84	±1.33
J		37.0	25.4	6.4	10.6
		22.3	26.3	6.3	9.2
		47.7	19.8	5.1	9.1
		52.3	16.2	11.0	16.7
		42.5	14.0	8.0	8.2*
	Mean	40.4	20.3	7.4	10.8
	S.E.M.	±5.19	±2.45	±1.02	±1.53

** See Table 17.

* Where the results are marked thus, the addition of urea made no appreciable difference to the amount of synthesis.

2. The highest values for protein nitrogen also occurred when the animals were grazing and the next highest when they were receiving Diet B which was made up of 10 lb. hay and 4 lb. concentrates of which about 40% consisted of bean meal and 14% of vetch meal. Where vetch meal was absent and the bean meal was present in lower proportions, as in Diets A and E, the values for protein nitrogen were also lower. 3. The level of N.P.N. varied considerably for any one diet. Thus with Diet E, for example, values ranging from 6.0 to 22.8 mg. N/100 g. rumen liquid were recorded.

It now remained to find whether the "synthesising power" of the centrifuged liquid was in any way related to the diet of the animals or to the N.P.N. and protein levels in the liquid. To investigate this point several of the samples, for which the nitrogen data are given in Table 18, were incubated with 1% maltose for 4 hours and the amount of synthesis determined. Each sample of liquid was divided into two portions; one was incubated with added urea (0.05%) and the other without it. The results are shown in Table 19. Taken as a whole they suggest (1) that the amount of synthesis tended to be greater when the animals were at pasture than when they were stall-fed, and (2) that the amount of synthesis for any one diet was not related to the level of either protein or N.P.N. in the liquid. Thus with Diet E, the synthesis given by one sample was equivalent to only 0.7 mg.N/100 g. rumen liquid whereas that given by the next sample was

equivalent to 5.5 mg. N/100 g. although the N.P.N. and protein levels were almost the same for both samples. Again for the grass diet (Diet J) the greatest and the least amounts of synthesis (11.0 and 5.1) were obtained with samples of similar protein and N.P.N. content.

In Tables 15, 16 and 19 results are recorded which show the effect of incubating rumen liquid in the absence and in the presence of extra N.P.N. added in the form of urea. It will be noted that in the great majority of the samples, but not in all of them, the synthesis appeared to be substantially increased in the presence of urea. Synthesis was measured by the decreases which occurred in the N.P.N. content of the liquid during incubation, and at first it was feared that the greater decrease, occurring when added urea was present, might be an artefact caused by some unknown factor. In all these experiments the N.P.N. was estimated after precipitation of protein with sodium tungstate in N/6 H_2SO_4, a process which would be expected to extract N.P.N. from rumen liquid after incubation just as completely as before it, and which should not, therefore, give rise to false values. It was realized by Pearson & Smith (1943, 1) that different methods of precipitating the proteins would probably give different N.P.N. values but it was believed that any one method used before and after incubation would give a reliable indication of the relative change in N.P.N. which had taken place. To test whether the method of estimating N.P.N. was likely to be at fault some incubations were carried out with

TABLE 20

The amount of synthesis occurring in samples of rumen liquid with and without added urea when estimated by two different methods

Sample	Sodium tungstate + N/6 H_2SO_4		Trichloroacetic acid	
	Without urea	With urea	Without urea	With urea
	(mg. N/100 g. rumen liquid)			
(a)	3.4	4.3	3.6	4.7
(b)	8.0	8.2	5.5	7.7
(c)	1.9	10.6	3.7	9.5

and without the addition of urea, and the N.P.N. estimated both by the tungstate method and by the trichloroacetic acid method described on p.20. Typical results are recorded in Table 20, where it will be seen that in one sample (a) adding urea made only a slight difference and the results by both methods were very much alike. In another sample (b) there was no change in the amount of synthesis on adding urea as determined by the tungstate method, whereas by the trichloroacetic acid precipitation the addition of urea appeared to result in a small increase. In sample (c) the amounts of synthesis, as estimated by the two methods, differed slightly in the absence of added urea, but by both methods urea was found to cause a very marked increase, i.e. from 1.9 and 3.7 to 10.6 and 9.5 mg. N/100 g. respectively. It is clear, therefore, that whatever the cause of the increased change during incubation when added urea is present, it is found to occur whether the N.P.N. is estimated by precipitating with sodium tungstate in N/6 H_2SO_4 or with trichloroacetic acid.

Furthermore should the apparent increase in synthesis which so often occurs on adding urea be an artefact it would be reasonable to expect it in samples which although incubated in the usual way proved to be inactive. Such a sample occurred in Experiment 4 in Table 15. Of the three samples used in that experiment, one taken one hour after feeding gave a small amount of synthesis, and a definite decrease in pH. The fasting sample gave no measurable amount of synthesis, but the

fact that the pH did tend to decrease suggests that it was capable of synthesis if conditions were favourable. With both these samples addition of urea brought about an increase in the amount of synthesis. In the third sample taken 4½ hours after feeding, however, both N.P.N. and pH changes indicated that synthesis had not occurred, and in this sample addition of urea did not affect the N.P.N. changes.

In view of the fact, however, that the amount of synthesis given by a sample of rumen liquid does not appear to be related to its "natural" level of N.P.N., the suggestion that it frequently gives increased synthesis when urea is added must be accepted for the present with reserve.

Summary

Experiments have been carried out to find whether the amount of synthesis given by a sample of rumen liquid is related to the concentration of protein or N.P.N. in the liquid and whether these concentrations are related to the type of rations fed.

The N.P.N. and protein levels and the amount of synthesis all appeared to be higher when the animals were at pasture than when they were stall-fed with hay and concentrates, but apart from this broad generalization, there appeared to be no marked relationship between the diet, the naturally occurring levels of protein and N.P.N. and the amount of synthesis.

When urea was added to rumen liquid the amount of synthesis usually appeared to be increased, but further work is required before it can be assumed with certainty that this apparent increase is real.

The "synthesising power" of rumen liquid has been found to be greatest just a short time after the fistula animals are fed. Other workers have found that bacterial counts and degree of fermentation are also greatest at that time.

Part IV The Effect of Aeration and of the Nature of the Carbohydrate present on the Amount of Protein Synthesis occurring in vitro

Introduction

In the previous section it was pointed out that the amount of synthesis obtained in a given time when rumen liquid was incubated *in vitro* varied greatly from sample to sample, and experiments were described in which a study was made of the effect of diet on the nitrogen partition in the rumen liquid and its "synthesising power". In earlier experiments, Pearson and Smith (1943, 3) found that utilization of N.P.N. with the formation of protein occurred only when a readily available form of carbohydrate was present in the rumen liquid. The carbohydrate could be soluble starch or one of the more common sugars such as maltose or glucose. It seemed probable that when concentrates are fed to a cow and the N.P.N. utilized, the most readily available source of energy for the bacteria would be starch, but since other constituents of plant tissues such as the pentoses of some of the polysaccharide complexes might have an important contribution to make, it was decided to determine whether pentoses can act as sources of energy and to find how they affect the "synthesising power" of the rumen liquid. This in turn led to experiments to determine what products were formed when

pentoses were utilized and what substances other than sugars could be used by the bacteria as sources of energy.

Another factor which might affect the amount of synthesis obtained was the type of gas present during an incubation. In the rumen, there is very little oxygen present, and in earlier work it had been found that when more than 150 mg. urea were added to 100 g. rumen liquid before incubation, the ammonia formed from the urea caused a considerable rise in pH and the amount of synthesis obtained was greatly reduced. Aerating the sample with CO_2 overcame these difficulties, but preliminary tests indicated that when the N.P.N. level was more within the normal range of 10 to 30 mg./100 g. rumen liquid, aeration with CO_2 was unnecessary. However, it was felt advisable to carry out some well controlled experiments to find to what extent the amount of synthesis was affected when the incubating liquid was aerated with different gases such as carbon dioxide, air, nitrogen and oxygen.

Experimental

1. Aeration Two experiments were made in which maltose (1%) and urea (0.04%) were added to two different samples of rumen liquid which had been strained through muslin and centrifuged. Each sample was divided into 5 portions. One portion was incubated in a flask covered with a watch-glass, and the contents of the flask were gently swirled round at intervals. The

TABLE 21

The effect of aerating the rumen liquid with different gases on the amount of synthesis produced

		Experiment 1		Experiment 2	
		pH	N.P.N. (mg.N/100g. rumen liquid)	pH	N.P.N. (mg.N/100g. rumen liquid)
No aeration	Initial	6.3	35.9	6.4	29.9
	Final	6.1	29.5	5.9	24.0
	Change	-0.2	Protein synthesis 6.4	-0.5	Protein synthesis 5.9
Carbon dioxide	Initial	6.3	35.9	6.4	29.9
	Final	6.0	30.7	5.8	27.1
	Change	-0.3	Protein synthesis 5.2	-0.6	Protein synthesis 2.8
Nitrogen	Initial	6.3	35.9	6.4	29.9
	Final	6.7	31.3	6.1	25.8
	Change	+0.4	Protein synthesis 4.6	-0.3	Protein synthesis 4.1
Air	Initial	6.3	35.9	6.4	29.9
	Final	7.1	32.0	6.4	29.0
	Change	+0.8	Protein synthesis 3.9	0.0	Protein synthesis 0.9
Oxygen	Initial	6.3	35.9	6.4	29.9
	Final	8.6	36.1	7.8	32.3
	Change	+2.3	Protein hydrolysis 0.2	+1.4	Protein hydrolysis 2.4

other 4 portions were incubated in flasks fitted with rubber bungs carrying an outlet tube and an inlet tube which dipped into the rumen liquid. The outlet tube of one of the flasks was connected to a water suction pump by which a steady stream of air was drawn through the liquid. The inlet tubes of the three remaining flasks were connected to cylinders of CO_2, nitrogen and oxygen. The N.P.N. content of the liquid was estimated initially and after incubation for 4 hours.

The results are recorded in Table 21. Reference to the values for the first experiment show that synthesis was greatest in the sample which was not aerated, 6.4 mg.N/100 g. rumen liquid. Aeration with CO_2 reduced the amount of synthesis slightly, while with nitrogen and air the inhibitory effects were still more marked. When oxygen was used no protein synthesis was recorded and the pH increased to the very abnormally high value of 8.6. In the second experiment the sample not aerated again gave the greatest synthesis, whereas on aeration with oxygen, protein hydrolysis predominated over protein synthesis. The pH changes in both experiments suggested that the fermentation was most active in the unaerated and CO_2 flasks and that it was reduced on aeration with nitrogen and still further reduced with air.

It seems, therefore, that the greatest bacterial activity occurs under conditions in which the rumen liquid is not aerated in any way but in which it is simply swirled round gently from time to time, in a

loosely stoppered flask, no special precautions being taken to exclude air. Presumably the CO_2 and CH_4 formed under these conditions maintain the most suitable atmosphere for the functioning of the bacteria. Indeed it is probable that these conditions are very similar to those existing in the intact rumen where only relatively minute amounts of air can enter from time to time as the animal swallows, and in which the formation of CO_2 and CH_4 maintains the bacteria under conditions which are practically anaerobic.

As a result of these experiments it was decided not to pass CO_2 or any other gas through the incubation flasks unless gas collection was required as in the experiments described on pp. 31-40. Even there the current of air used during incubation was very slow compared with that used in the aeration experiments which have just been described.

2. The value of various compounds as sources of energy for rumen bacteria It is obvious that the utilization of N.P.N. to form protein is dependent on the growth and multiplication of bacteria and this in turn will depend on an adequate supply of energy suitable for the bacteria to use. Energy supply is, therefore, almost certainly one of the most important factors governing the amount of synthesis. A few years ago Pearson and Smith (1943, 3) showed that soluble starch, galactose, sucrose, maltose, lactose and glucose could all be readily utilized by the rumen microorganisms, and

it was decided to extend this work to test the value of different starches, of some other sugars, of certain substances related to the sugars and of certain organic acids.

The method usually adopted in a typical experiment to test the value of a substance as a source of energy was as follows. A sample of rumen liquid strained through muslin and centrifuged was divided into the required number of portions. One was incubated for 4 hours with 1% maltose and another without the addition of any substance which might act as an energy supply. The remaining portions were incubated with 1% of the substance or substances which were to be tested. Urea (0.05%) was added to all the samples to ensure that the N.P.N. level was not so low as to become a limiting factor. In several of the experiments, protein synthesis was determined not only by observing the decrease which occurred in N.P.N., but also by isolating the bacterial sediment from samples of the rumen liquid by prolonged centrifuging before and after incubation and estimating the nitrogen which it contained as described on p.24. In the tests with different starches the changes in N.P.N. were determined not only before and after incubation but also at intervals during it.

(a) Starches Van der Wath (1942) working in South Africa, studied the rate of disintegration of starch granules from different cereals in the rumen of sheep. He found by in vivo experiments that the time required for complete digestion as determined by microscopical

TABLE 22

The amounts of protein synthesis obtained when rumen liquid was incubated with different starches and with maltose

Carbohydrate (1% of each added)	N.P.N. content of the rumen liquid (mg. /100 g.)				Protein synthesis calculated from the N.P.N. values (mg.N/100g. rumen liquid)		
	Initially	1 hr.	2 hr.	4 hr.	After 1 hr.	2 hr.	4 hr.
Maltose	39.3	37.9	35.7	30.4	1.4	3.6	8.9
Raw potato starch	37.1	37.6	37.2	34.2	-0.5	-0.1	2.9
Boiled potato starch	38.8	34.6	30.7	23.3	4.2	8.1	15.5
Raw maize starch	39.4	38.8	37.4	33.9	0.6	2.0	5.5
Boiled maize starch	38.4	34.8	32.2	22.6	3.6	6.2	15.8

examination, depended on the size of the starch granules. Potato starch granules (average diameter 35 microns) required 28 to 30 hours for complete digestion, whereas oat starch (average diameter of granules 6 microns) required only 17 to 20 hours and maize starch (12 microns) 18 to 22 hours. Since only soluble starch had been used so far in the present work, the value of raw potato and maize starch was now investigated.

Five portions of a sample of rumen liquid strained through muslin but uncentrifuged were incubated for 4 hours in the presence of 0.05% added urea. As a source of energy each portion of rumen liquid contained one of the following substances at a level of 1%:- maltose, raw maize starch, boiled maize starch, raw potato starch and boiled potato starch. Initially and after 1, 2 and 4 hours samples were removed for N.P.N. determinations. The results are shown in Table 22 and in Fig. 4. With raw potato starch the typical protein synthesis which is usually observed took some time to begin and even after 4 hours the amount of synthesis was slight. Raw maize starch gave much better results, but neither of the raw starches was so good as maltose. When, however, the starches were boiled before being added to the rumen liquid, the results obtained exceeded those given by 1% maltose. In the raw starch the size of the grain was probably important.

(b) Sugars other than pentoses In a typical experiment a sample of rumen liquid was divided into four portions. One was incubated for 4 hours with 1% maltose, the second

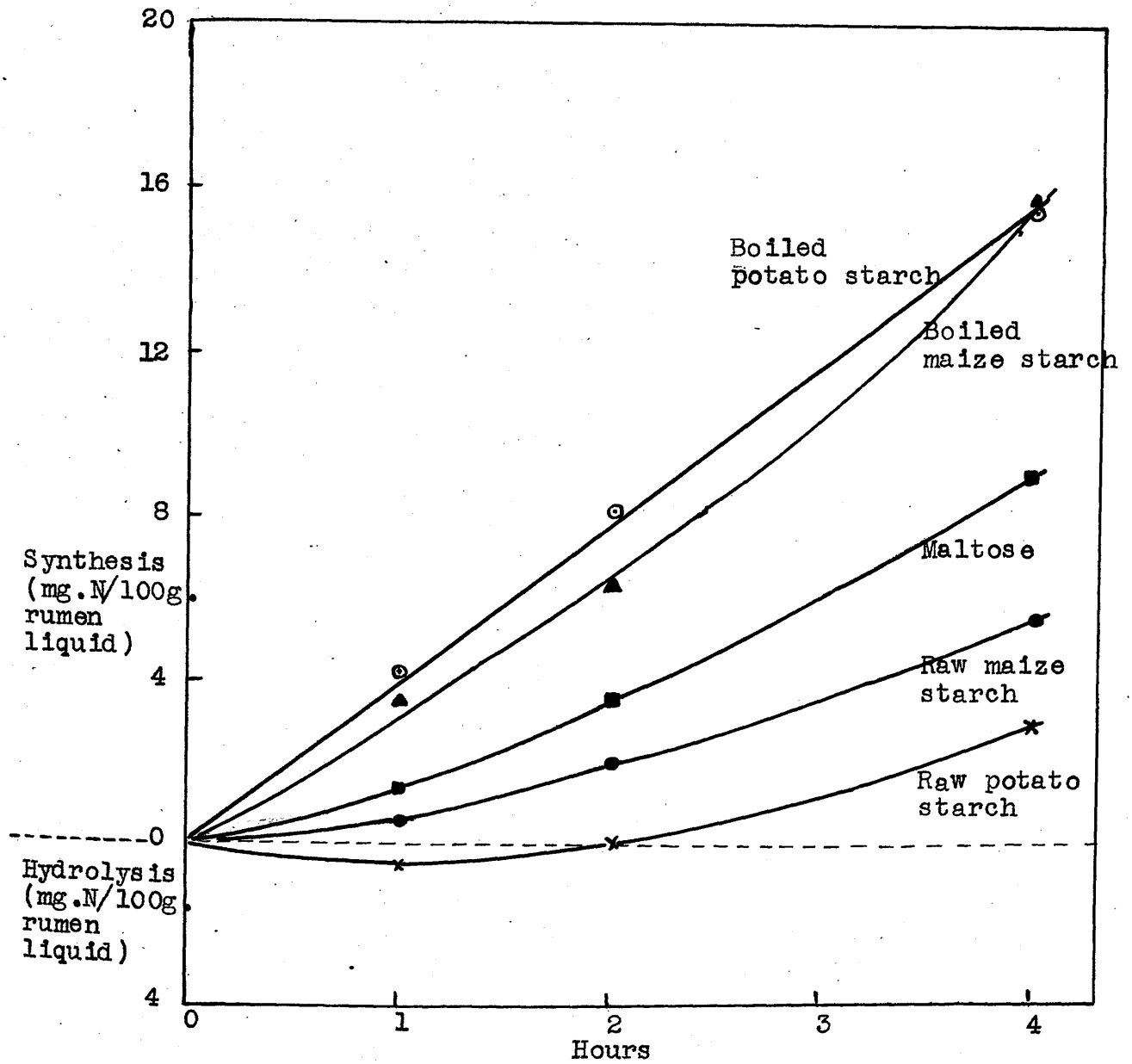


Figure 4. The amounts of protein synthesis obtained when rumen liquid was incubated with different starches and with maltose.

TABLE 23

The amount of protein synthesis obtained when fructose and cellobiose were used instead of maltose as sources of energy for rumen bacteria

Carbohydrate added	N.P.N. (mg.N/100g. rumen liquid)		Weight of bacterial sediment (mg./100g. rumen liquid)		Nitrogen of bacterial sediment (mg.N/100g. rumen liquid)	
No carbohydrate added	Initial	46.6	Initial	293	Initial	20.9
	Final	51.6	Final	196	Final	15.8
	Protein breakdown	5.0	Loss	97	Protein breakdown	5.1
Maltose	Initial	46.6	Initial	293	Initial	20.9
	Final	33.6	Final	522	Final	37.3
	Protein synthesis	13.0	Gain	229	Protein synthesis	16.4
Fructose	Initial	46.6	Initial	293	Initial	20.9
	Final	32.6	Final	500	Final	38.8
	Protein synthesis	14.0	Gain	207	Protein synthesis	17.9
Cellobiose	Initial	46.6	Initial	293	Initial	20.9
	Final	33.1	Final	539	Final	37.8
	Protein synthesis	13.5	Gain	246	Protein synthesis	16.9

without any added sugar, the third with 1% fructose and the fourth with 1% cellobiose. The results for this particular experiment are shown in Table 23, and will be discussed in detail at this stage so that the results for the remaining tests can be recorded more briefly. Not only was the N.P.N. estimated before and after incubation, but in this and several other experiments the weight and nitrogen content of the bacterial sediment were also determined as described on p.24.

It will be noted from the results in Table 23 that when no added carbohydrate was present, the weight of bacterial sediment decreased during incubation, whereas with each of the three sugars there was a considerable increase of 207 to 246 mg./100 g. This particular sample of rumen liquid was obviously very active and from the N.P.N. estimations it gave a synthesis of as much as 13.0 mg.N/100 g. rumen liquid with maltose and almost identical amounts with fructose and cellobiose. From the amount of nitrogen in the bacterial sediment which was isolated it was confirmed that protein breakdown had predominated in the absence of added carbohydrate, and that with the other three sugars, a considerable amount of synthesis occurred, the amount for all three sugars being practically the same. The value of the three sugars was, therefore, shown in three ways, (1) by the increase in the amount of bacterial sediment isolated, (2) by the nitrogen content of the sediment, and (3) by estimating the changes which occurred in N.P.N.

TABLE 24

The amount of protein synthesis obtained when mannose, sorbose, raffinose and inulin were used instead of maltose as sources of energy for rumen bacteria

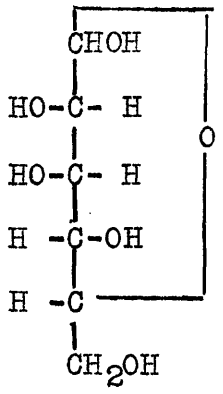
Experiment	Carbohydrate added (1%)	N.P.N. (mg. N/100g. rumen liquid)		Nitrogen of bacterial sediment (mg. N/100g. rumen liquid)
1.	No carbohydrate added	Initial	41.5	21.7
		Final	39.9	22.1
		Protein synthesis	1.6	0.4
	Maltose	Initial	41.5	21.7
		Final	32.0	31.8
		Protein synthesis	9.5	10.1
	Raffinose	Initial	41.5	21.7
		Final	34.1	29.0
		Protein synthesis	7.4	7.3
	Inulin	Initial	41.5	21.7
		Final	32.3	29.3
		Protein synthesis	9.2	7.6
2.	Maltose	Initial	50.3	42.6
		Final	44.6	48.9
		Protein synthesis	5.7	6.3
	Mannose	Initial	50.3	42.6
		Final	44.8	47.1
		Protein synthesis	5.5	4.5
	Sorbose	Initial	50.3	42.6
		Final	53.3	41.7
		Protein breakdown	3.0	0.9

The amount of synthesis which appeared to take place as indicated by the change in N.P.N. was 13.0 to 14.0 mg./100 g., whereas the corresponding values obtained by estimating the nitrogen of the bacterial sediment were 16.4 to 17.9 mg./100 g. The amounts by the two methods, therefore, were of the same general order but were not identical. This is undoubtedly due to experimental errors which are extremely difficult to avoid in the somewhat elaborate process of isolating the bacterial sediment and estimating its nitrogen content.

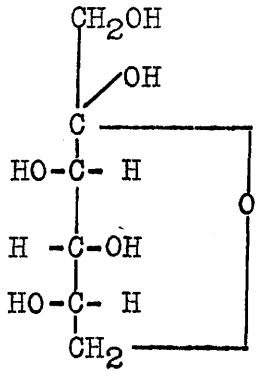
The results recorded in Table 23 show very clearly that fructose and cellobiose can be utilized by the rumen bacteria just as well as maltose.

Other sugars which were tested, apart from the pentoses, were the monosaccharide mannose, the ketohexose sorbose, the trisaccharide raffinose and the polysaccharide inulin (See Fig.5). Many of the other more common sugars and dextrin had already been tested by Pearson and Smith (1943, 3). The results for these four additional compounds are recorded in Table 24. In one experiment mannose and sorbose were compared with maltose, and in another experiment with a different sample of rumen liquid, raffinose and inulin were compared with maltose. The results in Table 24 show that raffinose, which contains units of fructose, glucose and galactose, and inulin, which consists of fructose units, were utilized, though possibly not quite as readily as maltose. The monosaccharide mannose was just as good as maltose within the limits of experimental error, whereas sorbose resulted in protein breakdown rather than synthesis and, therefore, did not appear to be utilized.

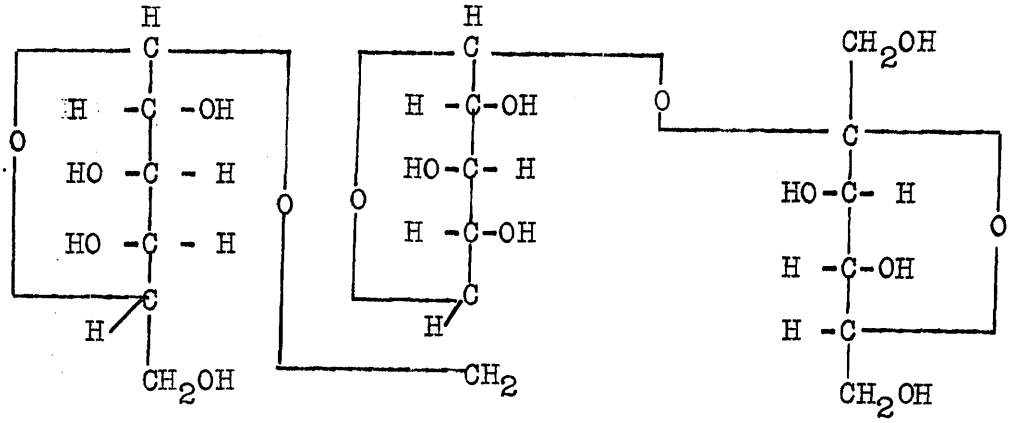
FIGURE 5



D (+) mannose

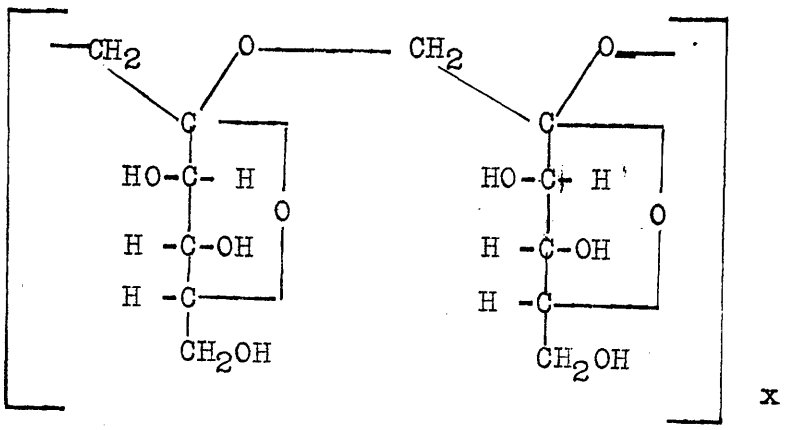


L (-) sorbose



Galactose Glucose Fructose

Raffinose



Inulin

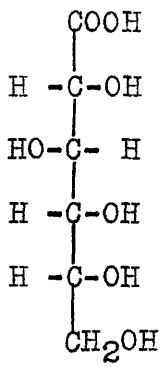
TABLE 25

The effect of sorbose before and after recrystallisation on the amount of protein synthesis obtained during the incubation of rumen liquid

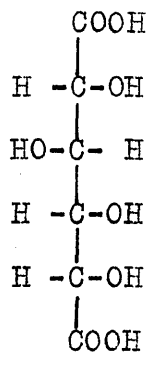
		N.P.N. (mg. N/100 g. rumen liquid)		
		Initial	Final	Protein synthesis (+) breakdown (-)
Before recrystalling the sorbose	1% Maltose	51.7	43.8	+ 7.9
	½% Maltose	45.4	40.5	+ 4.9
	½% Sorbose + ½% Maltose	42.7	41.7	+ 1.0
	½% Sorbose	46.2	49.3	- 3.1
After recrystalling the sorbose	1% Maltose	49.8	41.8	+ 8.0
	1% Sorbose (recrystallised)	54.7	55.2	- 0.5
	No carbohydrate	48.9	47.8	+ 1.1

The question naturally arose as to whether the absence of synthesis with sorbose was due to the configuration and nature of the sorbose molecule or whether it was due to the presence of some toxic impurity in the sorbose. Further incubation experiments with this sugar were, therefore, carried out, and in one of these additional experiments, samples of rumen liquid were incubated with 1% maltose, 0.5% maltose, 0.5% sorbose and with a mixture of 0.5% maltose and 0.5% sorbose. The results are shown in Table 25. As has been found previously (Pearson and Smith, 1943, 3) 0.5% maltose gave less synthesis than 1% maltose, 4.9 as compared with 7.9 mg.N/100 g. When, however, 0.5% sorbose was used along with 0.5% maltose, protein breakdown predominated over synthesis. This suggested that some toxic agent might be present in the sorbose, since if the sorbose was harmless, the 0.5% maltose would still have been expected to produce synthesis of about 4.9 mg.N/100 g. rumen liquid even in the presence of sorbose. The sample of sorbose was found to have a melting point of 157°C as compared with the value of 165°C recorded in the literature. Attempts were made, therefore, to purify it by recrystallization with charcoal which resulted in the melting point rising to 159°C, but as shown in Table 25 the recrystallized sample still caused protein breakdown rather than synthesis, the addition of the recrystallized sorbose giving a result inferior to that obtained with the sample to which no addition was made. It is still

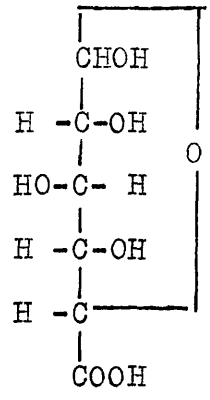
FIGURE 6



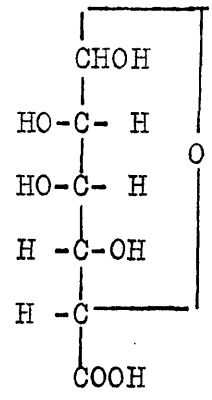
Gluconic acid



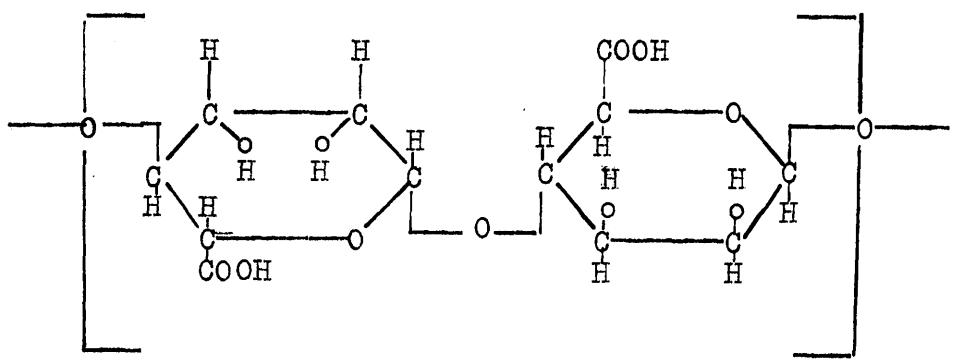
Saccharic acid



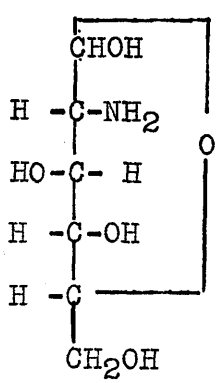
Glucuronic acid



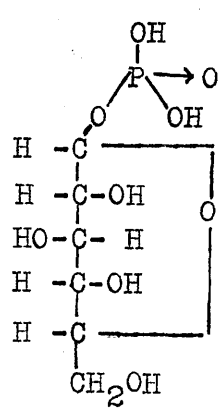
Mannuronic acid



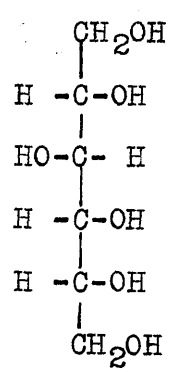
Alginic acid



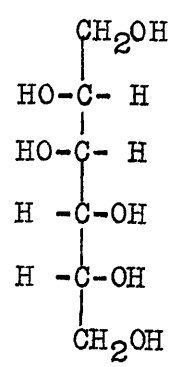
Glucosamine



Glucose-1-PO_4



D-sorbiotl



D-mannitol

doubtful, therefore, whether this effect of sorbose is due to its particular type of structure or to the presence of a toxic impurity. Sorbose, a ketohexose, is not known to occur naturally except as the bacterial oxidation product of the hexahydric alcohol, sorbitol, found in rowan berries, so that it is not of practical importance. The object of testing it was simply to gather information as to the types of compound and the type of molecular configuration on which the rumen bacteria can act.

(c) Substances related to carbohydrates Many substances structurally related to the carbohydrates, such as the hexahydric alcohols, the sugar acids, uronic acids, aldonic acids and amino-sugars, occur naturally and it was of interest, therefore, to find out whether any of these compounds could be utilized by the rumen bacteria. It was also hoped that by studying some of these compounds, further information might be obtained as to the types of molecular structure on which the rumen bacteria can act.

The scope of this section of the work was somewhat limited by the supply of carbohydrate derivatives available. Professor M. Stacey of Birmingham University, however, very kindly supplied samples of gluconic acid, glucuronic acid, saccharic acid, glucosamine and glucose-1-phosphate. These were tested in addition to mannitol, sorbitol and alginic acid, a polymer of mannuronic acid units. For the chemical formula of most of these compounds reference should be made to Fig. 6.

TABLE 26

A brief summary of the results obtained in incubations of rumen liquid when maltose or glucose was replaced by substances related to the carbohydrates

Expt. No.	Control		Experimental	
	Sugar added (1%)	Amount of protein synthesis (+) or breakdown (-) (mg.N/100g. rumen liquid)	Substance being tested (1%)	Amount of protein synthesis (+) or hydrolysis (-) (mg.N/100g. rumen liquid)
1	Maltose	+ 7.0	Gluconic acid	- 1.0
	Glucose	+ 7.1	Glucuronic acid	- 0.6
			Saccharic acid	- 0.2
2	Maltose	+ 5.7	Mannitol	+ 0.6
			Sorbitol	+ 0.6
3	Glucose	+ 7.0	Glucosamine	+ 0.7
			Glucose-1-PO_4	+ 11.1
4	Maltose	+ 3.6	Alginic acid	+ 0.9

The results of this work are shown in Table 26. Of the substances tested, glucose-1-phosphate was the only one with which any significant amount of synthesis was obtained. It appeared to be more efficient than glucose itself, which might suggest that in the rumen as in other types of fermentation, phosphorylation is an essential part of the process, but since only enough of the ester was available for one test, this result required further confirmation before definite conclusions may be drawn.

It may be inferred from the negative results with the other substances that the rumen bacteria require compounds like the sugars which possess a potential aldehyde or ketone group together with a primary alcohol group, or substances like sucrose and polysaccharides which can readily give rise to compounds of this type. For instance, glucose is readily utilized, but oxidation of glucose to gluconic, glucuronic or saccharic acid renders it unfermentable. Similarly, reduction of the aldehyde group to form a hexitol stops fermentation as instanced by the activity with mannose and inactivity with mannitol.

It had been suggested that addition of N.P.N. to feedingstuffs might be conveniently done by using ammonium alginate, thus adding N.P.N. in combination with a carbohydrate derivative which might supply energy, but alginic acid proved to be of no value, and as a result of these tests the idea was abandoned.

(d) Volatile fatty acids The importance of the volatile fatty acids, acetic, propionic and butyric as

TABLE 27

The absence of protein synthesis when maltose was replaced by the sodium salts of the volatile fatty acids and by sodium β-hydroxy butyrate

Expt. No.	Substance added	N.P.N. (mg.N/100 g. rumen liquid)		Weight of bacterial sediment (mg./100g. rumen liquid)		Nitrogen of bacterial sediment (mg.N/100g. rumen liquid)	
1	No addition	Initial	46.6	Initial	293	Initial	20.9
		Final	51.6	Final	196	Final	15.8
		Protein breakdown	5.0	Loss	97	Protein breakdown	5.1
	Maltose	Initial	46.6	Initial	293	Initial	20.9
		Final	33.6	Final	522	Final	37.3
		Protein synthesis	13.0	Gain	229	Protein synthesis	16.4
	Sodium propionate	Initial	46.6	Initial	293	Initial	20.9
		Final	44.3	Final	280	Final	20.3
		Protein synthesis	2.3	Loss	13	Protein breakdown	0.6
2	No addition	Initial	44.1	Initial	167	Initial	11.6
		Final	45.7	Final	130	Final	8.9
		Protein breakdown	1.6	Loss	37	Protein breakdown	2.7
	Maltose	Initial	44.5	Initial	151	Initial	9.9
		Final	37.3	Final	324	Final	20.1
		Protein synthesis	7.2	Gain	173	Protein synthesis	10.2
	Sodium acetate	Initial	41.6	Initial	224	Initial	13.7
		Final	41.2	Final	178	Final	11.6
		Protein synthesis	0.4	Loss	46	Protein breakdown	2.1
	Sodium butyrate	Initial	41.6	Initial	224	Initial	13.7
		Final	42.9	Final	196	Final	11.5
		Protein breakdown	1.3	Loss	28	Protein breakdown	2.2
3	No addition	Initial	41.5	Initial	356	Initial	21.7
		Final	39.9	Final	330	Final	22.1
		Protein synthesis	1.6	Loss	26	Protein synthesis	0.4
	Maltose	Initial	41.5	Initial	356	Initial	21.7
		Final	32.0	Final	520	Final	31.8
		Protein synthesis	9.5	Gain	164	Protein synthesis	10.1
	Sodium β-hydroxy butyrate	Initial	41.5	Initial	356	Initial	21.7
		Final	38.0	Final	325	Final	23.1
		Protein synthesis	3.5	Loss	31	Protein synthesis	1.4

products of fermentation in the rumen has been fully stressed within recent years, mainly as a result of the work which has been reviewed recently by Phillipson (1947-48). Little is known, however, as to whether the rumen bacteria can act further on these substances or whether they are to be regarded solely as end-products. Indeed, by in vivo experiments in the rumen itself, it would be difficult to gain information on this point, since any decrease in the content of volatile fatty acids occurring in the rumen ingesta might all be due to absorption of the acids into the blood-stream or their passage further along the alimentary tract. The present in vitro methods of study provided, therefore, a means of ascertaining whether these volatile fatty acids were capable of acting to any measurable extent as sources of energy for the bacteria. A test was also made with β-hydroxybutyric acid. The technique employed was the same as that described on p.82 for testing the value of fructose and cellobiose. The results are recorded in Table 27. The rumen liquid used in each of the three experiments was shown to be active by the fact that on incubating it in the presence of maltose the N.P.N. decreased, the bacterial sediment increased very markedly, and the nitrogen in the sediment also increased. But when in Experiment 2, maltose was replaced by acetate and butyrate, the changes differed very little indeed from those occurring when no addition was made to the rumen liquid. It is clear, therefore, that acetic and butyric acids were not used to any measurable extent.

TABLE 28

Protein synthesis and change in lactic acid content on incubating rumen liquid with lactic acid and maltose

Expt. No.	Substance added		pH	N.P.N. (mg.N/100g. rumen liquid)	Lactic acid (mg./100g. rumen liquid)
	Maltose	Lactic acid			
1	1%	None	Initial 6.4 Final 5.5 Change -0.9	Initial 49.6 Final 40.4 Protein synthesis 9.2	Initial 8 Final 199 Increase 191
	1%	0.5% (Approx.)	Initial 6.0 Final 5.6 Change -0.4	Initial 49.6 Final 44.8 Protein synthesis 4.8	Initial 581 Final 667 Increase 85
	None	0.5% (Approx.)	Initial 6.0 Final 6.9 Change +0.9	Initial 47.1 Final 47.6 Protein breakdown 0.5	Initial 585 Final 561 Decrease 24
2	1%	None		Initial 30.5 Final 23.3 Protein synthesis 7.2	Initial 4 Final 106 Increase 102
	1%	0.1% (Approx.)		Initial 26.7 Final 17.9 Protein synthesis 8.8	Initial 107 Final 220 Increase 113
	None	0.1% (Approx.)		Initial 26.6 Final 28.2 Protein breakdown 1.6	Initial 106 Final 103 Decrease 3

From the results for weight and nitrogen content of the sediment in the third experiment, it is probable that β-hydroxy butyric acid, like butyric acid itself, was not utilized to any extent. From the results of the first experiment, however, it appears that propionic acid may have been able to act as a source of energy, though to a very limited degree compared with maltose. Thus by all three methods of measuring the activity, the results for sodium propionate fell between those for maltose and those for no addition, but they were decidedly nearer the latter. The amount of protein breakdown and the decrease in the nitrogen of the bacterial sediment were definitely smaller in the presence of propionate than they were when no addition whatever was made.

(e) Lactic acid The technique which has just been described was now applied to determine whether lactic acid is likely to be a true end-product of fermentation in the rumen or whether it can be utilized by the bacteria to any measurable extent. In the first experiment (Table 28) a sample of rumen liquid was divided into three portions. One portion was incubated with 1% maltose, another with 1% maltose plus 0.5% lactic acid, and the third with 0.5% lactic acid and no maltose. (The lactic acid was partially neutralized with NaOH). In the presence of maltose, 0.5% lactic acid gave less protein synthesis than maltose alone. A concentration of 0.5% lactic acid is much greater than would ever exist in practice and so in the second experiment, the

TABLE 29

A brief summary of the results obtained in incubations of rumen liquid when maltose was replaced by a number of different organic acids

Expt. No.	Control		Experimental	
	Sugar added	Amount of protein synthesis (+) or hydrolysis (-) mg.N/100 g. rumen liquid	Substance tested (1%)	Amount of protein synthesis (+) or hydrolysis (-) (mg.N/100 g. rumen liquid
1	Maltose 1%	+14.1	Sodium citrate	-2.7
			Potassium hydrogen malate	-1.2
	No addition	- 2.2	Sodium potassium tartrate	-1.3
			Fumaric acid	-0.5
2	Maltose (0.3%)	+ 2.3	Succinic acid (0.3%)	-2.4

amount of lactic acid added was reduced to 0.1%. In this smaller amount it was found to have no inhibiting effect whatever on synthesis in the presence of maltose. In both experiments when lactic acid was used alone, protein breakdown predominated rather than synthesis, and the decrease which occurred in the lactic acid content of the two rumen liquids was only about 3 or 4% of the total lactic acid present, and this is probably just about equal to the experimental error which might reasonably be expected. It seems, therefore, that lactic acid is not utilized for the bacterial growth which results in protein synthesis, and that it is almost certainly a true end-product which is not decomposed further in the rumen. A few years ago Elsden (1945-46) found that when certain rumen bacteria were incubated in synthetic medium for several days, lactic acid was converted to volatile fatty acids, but this did not occur in the present experiments where the mixed rumen flora was incubated in rumen liquid under conditions which were probably very similar to those existing in the normal rumen.

(f) Other organic acids A number of other organic acids, such as citric and succinic, were tested by the same technique. The results are shown in Table 29. None of the substances tested appeared able to act as a source of energy for the bacteria.

(g) The pentoses In many natural feedingstuffs pentoses exist in the form of complex polysaccharides. Thus when dried grass was analysed by the furfural-

TABLE 30

A preliminary test to determine the value of pentoses as a source of energy for rumen bacteria

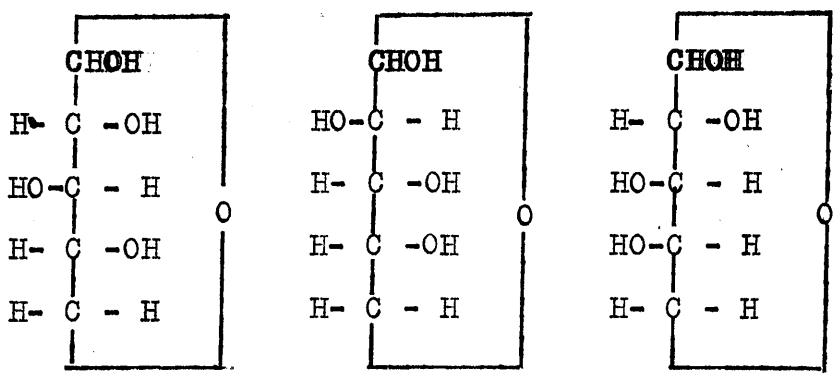
Carbohydrate added (1%)	Change in pH during each incubation (The initial pH was 6.85)	Amount of protein synthesis measured by decrease in N.P.N. (mg.N/100g. rumen liquid)	Increase in bacterial sediment (mg.100 g. rumen liquid)
No addition	+0.1	2.4	3
Maltose	-1.4	17.7	225
D(-)arabinose	0.0	4.4	18
L(+)arabinose	-0.4	9.9	188
D(+)xylose	-0.4	10.8	125

phloroglucide method it was found to contain the equivalent of 11.4% pentose. Bean meal and bruised oats gave values of 4.3 and 10.2%. It follows, therefore, that pentoses may have a significant, though possibly small part to play in the metabolism of the rumen bacteria. For this reason and also because the results might give further information on the types of compound which the rumen bacteria can utilize, it was decided to make a study of the pentoses as possible sources of energy for the bacteria. The only pentoses which were available in sufficient amounts during the course of the work were D(-)arabinose, L(+)arabinose and D(+)xylose. The formulae of these compounds is shown in Figure 7.

In a preliminary test, a sample of rumen liquid was divided into 5 portions. One was incubated without any addition, a second with maltose, and the remaining three with the three pentoses, all at an initial level of 1%. It will be seen from the results summarised in Table 30 that in this preliminary test, L(+)arabinose and D(+)xylose caused a considerable amount of bacterial activity to take place, though not so much as was obtained with maltose, and that D(-)arabinose gave very little activity particularly as judged from the change in pH and the figures for the increase in bacterial sediment.

This lack of activity with D-arabinose might be due either to the fact that the bacteria could not utilize this particular isomer which occurs very rarely in nature, or because the sample of D-arabinose was

Figure 7



D(+)xylose D(-)arabinose L(+)arabinose

TABLE 31

The amount of protein synthesis obtained by incubating rumen liquid with pentoses alone and also with pentoses in the presence of maltose

Expt. No.	Carbohydrate added	Change in pH during incubation (Average initial value was 6.1)	Amount of synthesis(+) or hydrolysis(-) as estimated by change in N.P.N. during incubation (mg.N/100g. rumen liquid	Increase(+) or Decrease(-) in bacterial sediment during incubation (mg./100g. rumen liquid	Amount of synthesis(+) or hydrolysis(-) estimated by the change in the nitrogen content of the bacterial sediment (mg.N/100g. rumen liquid)
1	No carbohydrate	+0.7	-2.8	- 30	- 2.8
	Maltose 1%	-0.3	+ 7.2	+190	+ 7.3
	Maltose 0.5%	-0.3	+ 6.5	+153	+ 6.6
	L-arabinose 0.5% Maltose 0.5%	-0.4	+ 8.1	+204	+ 7.2
	L-arabinose 0.5%	0.0	+ 4.5	+108	+ 4.4
2	No carbohydrate	+0.4	- 1.0	- 29	- 1.9
	Maltose 1%	-1.1	+10.4	+309	+12.1
	Maltose 0.5%	-0.9	+ 6.8	+196	+ 7.5
	Maltose 0.5% D-arabinose 0.5%	-1.0	+ 9.3	+224	+10.2
	D-arabinose 0.5%	+0.4	+ 0.1	- 11	- 0.8
3	No carbohydrate	+0.5	- 1.2	- 29	- 1.6
	Maltose 1%	-0.7	+11.4	+328	+12.7
	Maltose 0.5%	-0.6	+ 5.9	+156	+ 6.5
	Maltose 0.5% D-xylose0.5%	-0.8	+10.9	+267	+11.1
	D-xylose0.5%	-0.3	+ 8.7	+155	+ 7.6

contaminated with some toxic material which inhibited bacterial growth. A second and more elaborate experiment was, therefore, planned in order to investigate the matter further. Each of the three pentoses was incubated in rumen contents alone to confirm or refute the first test, and also with maltose to determine, particularly with D-arabinose, whether the presence of pentose inhibited the activity resulting from maltose alone. As in some of the previous experiments (e.g. Table 23), the relative amount of activity was noted in three ways, (1) by the decrease in N.P.N. which occurred during incubation, (2) by the increase in the weight of the bacterial sediment, and (3) by the increase in the nitrogen of the bacterial sediment. The results are recorded in Table 31. They confirmed the results of the previous test in showing that L-arabinose and D-xylose were readily utilized by the rumen bacteria, while D-arabinose was not. Mixtures of 0.5% maltose with either 0.5% L-arabinose or 0.5% D-xylose resulted in just about as much protein synthesis as 1% maltose alone. The sample of D-arabinose clearly contained no toxic material, for on using 0.5% maltose in the presence of 0.5% D-arabinose, protein synthesis was in no way inhibited, but even appeared to be enhanced. It is not known why D-arabinose, which was so inactive by itself, should cause an increase in activity when mixed with maltose. It may be that once the series of reactions involved in the fermentation process is begun in the

presence of maltose, this particular isomer of arabinose can be used for some of the later reactions or for some of the processes involved in bacterial growth and thus have what would virtually be equivalent to a "sparing effect" on maltose.

3. The fermentation products formed when pentoses are utilized In the experiments with pentoses which have just been described, samples of the various rumen liquids before and after incubation were preserved with formalin and later examined by Mr. Frank Baker. He found that the bacterial sediment obtained after incubation from any sample of rumen liquid which had contained maltose always stained blue with iodine, indicating the presence of a starch-like polysaccharide in the iodophile bacteria (Baker, 1943), whereas when the liquid had contained pentoses and no maltose, the iodine test was negative, in spite of the fact that with two of the three pentoses fermentation and bacterial growth had certainly occurred. Since these observations were made a thesis by Van der Wath (1942) became available. It is of interest to note that he found that when rumen liquid was incubated with 2% or 5% of the pentoses, xylose, arabinose (optical isomer not specified) or rhamnose, the microorganisms did not form any iodophilic polysaccharide. He did, however, find when testing the fermentation characteristics of the iodophilic streptococcus from sheep rumen ingesta that arabinose was fermented whereas rhamnose was not. Xylose apparently was not tested. These findings lend support

TABLE 32

The increase in the polysaccharide of the bacterial sediment obtained after incubation of rumen liquid with different amounts of maltose and xylose

	Polysaccharide present	Increase in Polysaccharide
	(mg./100 g. rumen liquid)	
Rumen liquid before incubation	17.5	
Rumen liquid after incubation with:-		
1% maltose	196.8	+ 179.3
0.5% maltose	118.0	+ 100.5
0.5% maltose + 0.5% xylose	183.4	+ 165.9
0.5% xylose	68.9	+ 51.4

TABLE 33

The analysis of the bacterial sediment obtained before and after incubation of samples of the same rumen liquid with maltose and L-arabinose

	The composition of the bacterial sediment (%) obtained with the sugar shown below			
	Maltose		L-arabinose	
	Before incubation	After incubation	Before incubation	After incubation
Protein	55.6	39.3	57.8	39.1
Polysaccharide*	18.7	43.1	17.3	43.5
Pentose**		4.1		4.1

* Polysaccharide which was readily hydrolysed by dilute HCl.

** Probably present as nucleic acids.

TABLE 34

The analysis of the bacterial sediment obtained after incubation with maltose and with xylose

	The composition of the bacterial sediment (%) obtained with the sugar shown below			
	Experiment 1		Experiment 2	
	Maltose	D-xylose	Maltose	D-xylose
Protein	37.4	43.7	41.2	45.3
Polysaccharide*	45.8	35.4	45.2	37.3
Pentose**	2.7	2.2	2.9	3.4

* Polysaccharide which was readily hydrolysed by dilute HCl.

** Probably present as nucleic acids.

to the conclusion drawn from the present observations that when a pentose is utilized by the bacteria, the carbohydrate built up or stored in the bacterial cells is quite different from that stored when ordinary hexoses or compounds containing hexose units are used. That polysaccharide is certainly formed when xylose is utilized, but to a much smaller extent than when maltose is the source of carbohydrate, is shown by the results recorded in Table 32. In this experiment, there was not sufficient of the bacterial sediment available to determine whether the polysaccharide was pentosan in nature, but in later experiments with both L-arabinose and D-xylose, the bacterial sediment was prepared in amounts sufficient for the content of protein, readily hydrolysable polysaccharide and pentose to be estimated. The results for such an experiment, in which the composition of the bacterial sediment obtained with maltose was compared with that of the sediment obtained with L-arabinose, are shown in Table 33. It is clear that the sediments obtained after incubation were practically the same whether the sugar utilized was maltose or L-arabinose, and that the polysaccharide formed during incubation with pentose did not contain any higher proportion of pentose units. Corresponding results were obtained for the sediment after incubation in two further experiments using xylose instead of arabinose. The results are recorded in Table 34. In these experiments, the sediment obtained with xylose contained more protein and less polysaccharide than the

TABLE 35

The amounts of the various products formed during incubation with arabinose, and the amount of the utilized arabinose which was accounted for by the formation of these products

	The amount of substance formed or decomposed during incubation (mg./100 g. rumen liquid)	The percentage of the utilized arabinose, expressed as carbon, which is accounted for by the carbon of each product.
Amount of sugar changed	375	100
Volatile fatty acids produced (as acetic acid)	120	32.0
Lactic acid	2	0.5
Carbon dioxide	22	4.0
Methane	2	1.0
Bacterial protein	50	17.7
Bacterial polysaccharide (expressed as starch)	64	18.9
Total accounted for		74.1

TABLE 36

The proportion of the fermented arabinose, expressed as carbon, which was accounted for by the carbon of the various end-products of fermentation

	The percentage of the arabinose assumed to be fermented* accounted for by each product, calculated on the basis of carbon
Volatile fatty acids (as acetic)	50.5
Lactic acid	0.8
Carbon dioxide	6.3
Methane	1.6
Unaccounted for	40.8
Total	100.0

* Namely, the total arabinose changed during incubation, as recorded in Table 35, less the amount utilized for the formation of bacterial protein and polysaccharide.

sediment obtained with maltose, but again it was clear that the polysaccharide formed when pentose was utilized did not possess a higher proportion of pentose units. In fact it is probable that the pentose contained in all these bacterial sediments, was present in the form of nucleic acid and not as polysaccharide (p. 54). As stated above, however, the polysaccharide formed when pentose is fermented differs from that formed when hexoses are fermented, in that it does not stain blue with iodine.

In part I, p.33, a description is given of the method used to determine the amounts of various substances formed when maltose was utilized by the rumen bacteria. In the present section of the work, it was decided to apply this method to a study of the products formed when pentoses were utilized. Time did not permit of an extensive investigation being made, but preliminary experiments were carried out with L-arabinose and D-xylose. Since the method of attacking this type of problem and of arriving at the results has already been given so fully on pp.33-39 and in Tables 6, 7 and 8, it will only be necessary here to summarise the results as in Tables 35 and 36. The corresponding results recorded in Tables 7 and 8 for three experiments with maltose were obtained at a different time with entirely different samples of rumen contents, but they may be used for comparing the fermentation of maltose with that of arabinose. It will be noted that with maltose (Table 7) 81 to 90% of the utilized maltose was accounted

for, whereas with arabinose the corresponding figure was only 74% (Table 35), and when an attempt was made to account for the arabinose actually fermented, the various products which were studied accounted for only about 59% (Table 36) as compared with 70 to 80% for maltose (Table 8). It will also be observed that although the volatile fatty acid formation compared well with that observed with maltose, the production of carbon dioxide and methane tended to be less, and the amount of lactic acid produced was extremely small. The very low production of lactic acid from pentose was confirmed by a similar experiment with xylose. In this experiment samples of the rumen liquid were also incubated with maltose. The hexose gave 155 mg. volatile fatty acids and 83 mg. lactic acid per 100 g. rumen liquid during incubation, whereas xylose gave almost as much volatile fatty acids (143 mg.) but only 8 mg. lactic acid.

It may be concluded that in the fermentation of pentoses, lactic acid is formed in only very small amounts and that a large proportion of the fermented pentose, amounting to some 40%, still remains unaccounted for. It is hoped to carry out further work when accurate partition of the volatile fatty acids is possible, and in that further work attempts will be made to find whether dibasic organic acids and other substances are formed in significant amounts when pentoses are fermented.

TABLE 37

The substance tested in earlier work and in the present experiments, showing those which can and those which cannot be utilized by the rumen bacteria

Substances with which protein synthesis was obtained		Substances with which protein synthesis was not obtained	
Type of substance	Name of substance	Type of substance	Name of substance
Polysaccharide	Starch Inulin Dextrin*	Compounds related to the hexoses	Alginic acid Glucosamine Gluconic acid Glucuronic acid Saccharic acid Mannitol Sorbitol
Trisaccharide	Raffinose		
Disaccharides	Cellobiose Lactose* Maltose Sucrose*	Trihydric alcohol	Glycerol*
Hexoses	Fructose Galactose* Glucose Mannose	Volatile fatty acids	Acetic Butyric
		Hydroxy-acid	β-Hydroxy butyric acid
Hexose phosphate	Glucose-1-PO_4	Other organic acids	Citric acid Lactic acid Malic acid Succinic acid Tartaric acid
Pentoses	L(+)Arabinose D(+)Xylose	Hexose	Sorbose**
		Pentose	D(-)Arabinose

When propionic acid was tested it appeared possible that it promoted a small amount of protein synthesis.

* These substances were tested in the earlier work of Pearson & Smith (1943, 3).

** The inactivity observed with sorbose may possibly have been due to the presence of some toxic impurity.

Summary and Conclusions

Tests have been made to determine whether the amount of protein synthesis occurring *in vitro* could be increased by aeration with carbon dioxide, nitrogen, air or oxygen. It was found that aeration with oxygen completely inhibited synthesis, and that aeration with the other gases caused no increase in synthesis. It is probable that incubating the rumen liquid with occasional gentle agitation in a flask not tightly closed resembles the conditions in the rumen as closely as any other gaseous system which can be devised.

For protein synthesis to occur, some source of energy such as starch or maltose must be present. A number of substances have now been tested to determine what type of compound will act as a source of energy for the bacteria. The results are summarised in Table 37. Hexoses, or substances which readily give hexoses on hydrolysis, were readily utilized, the only exception being sorbose which may have contained some toxic impurity. Hexitols and carboxylic acid derivatives of the hexoses were not utilized. Since glucuronic acid was one of the compounds not used, it would appear probable that the rumen bacteria require compounds having both an aldehyde and a primary alcohol group in addition to secondary alcohol groups.

L(+)arabinose and D(+)xylose gave some protein synthesis, but D(-)arabinose did not. This clearly indicated that the configuration of the molecule is an

important factor in determining whether the bacteria can utilize a compound. In this connection it is interesting to note from Figure 7 that the arrangement of hydrogen atoms and hydroxyl groups on the carbon of the potenial aldehyde group and on the two carbon atoms next to it is the same in L(+)arabinose as it is in D(+)xylose whereas in D(-)arabinose it is different.

Lactic, acetic and butyric acids did not appear to be utilized by the rumen bacteria. Propionic acid may have promoted some synthesis, but if so, the amount was very small.

The other organic acids tested were not utilized.

Some preliminary experiments were carried out to determine the amounts of various substances formed when pentoses were fermented. Compared with the corresponding results for maltose (Part 1), the formation of volatile fatty acids was normal, but the amount of lactic acid produced was very small. About 40% of the fermented pentose remained unaccounted for (Table 36) compared with 20 to 30% when maltose was used (Table 8).

Part V. The Effect of certain Metals on the Amount of Protein Synthesis obtained in vitro

Introduction

Within recent years, many advances have been made in the knowledge of the mineral requirements of cattle and sheep, mainly through the study of deficiency diseases. Such diseases as bush-sickness (New Zealand) and pining (Britain) have been shown to be due primarily to lack of cobalt in the pasture. Pastures deficient in copper are now recognised to be responsible for diseases such as swayback in lambs and falling disease in cattle. These diseases can be controlled by supplying cobalt or copper, or ferric salts containing cobalt and copper, directly to the animals or as top dressings to the pasture. There is, therefore, the possibility that variable amounts of these metals may at times be introduced into the rumen. Apart from experiments with copper such as those of Becker (1929) in which large doses of copper salts were used to destroy the protozoa of the paunch for experimental purposes, little is known of the effect which amounts of various metals in excess of requirements might have on the animal and on the activity of the bacteria in its rumen.

The presence of unusually large amounts of molybdenum in the "teart" pastures of Somerset is held responsible for scouring, and since administration of

TABLE 38

The effect of copper on the amount of protein synthesis obtained during the incubation of rumen liquid with 1% maltose

Expt. No.	Treatment of the rumen liquid before incubation	Substance added	Concentration of the metal in the rumen liquid	Synthesis (+) or hydrolysis (-) (mg.N/100g. rumen liquid
1	Strained through muslin and centrifuged for 4 minutes at 2,000 r.p.m.	Na as Na_2SO_4	1 in 20,000	+12.0
		Cu as $CuSO_4$	(1 in 100,000	+ 0.9
			(1 in 80,000	+ 0.6
			(1 in 70,000	+ 1.6
			(1 in 40,000	+ 0.8
			(1 in 20,000	+ 1.9
2	Strained through muslin and centrifuged for 2 minutes at 2,000 r.p.m.	Na as Na_2SO_4	(1 in 100,000	+11.7
			(1 in 2,000	+10.6
		Cu as $CuSO_4$	(1 in 100,000	+10.2
			(1 in 10,000	- 1.6
			(1 in 1,000	- 0.1
3	Strained through muslin but not centrifuged	Control	-	+ 7.0
		Na as Na_2SO_4	1 in 20,000	+ 6.4
		Cu as $CuSO_4$	(1 in 100,000	+ 7.1
			(1 in 80,000	+ 2.8
			(1 in 70,000	+ 2.3
			(1 in 60,000	+ 1.8
			(1 in 50,000	+ 0.4
			(1 in 40,000	- 3.1
			(1 in 30,000	- 1.4
			(1 in 20,000	- 5.3

copper can prevent scouring on such pastures, it is generally believed that the condition is due to copper deficiency caused by excewsive amounts of molybdenum interfering with copper metabolism. As scouring is evidence of digestive disturbances, the possibility that excess molybdenum might interfere with bacterial digestion in the rumen seemed to merit investigation. It was decided, therefore, to make some tests with copper, cobalt and molybdenum in an attempt to find to what extent they affected protein synthesis. Iron and lead were also tested.

Experimental

The method adopted was to add 1% maltose and 0.05% urea to a sample of rumen liquid and divide it into several portions. One portion was incubated without any further addition and the others with various concentrations of salts of the metal whose effect was being studied. The amount of protein synthesis as measured by the decrease in N.P.N. was taken as an indication of the effect of the metal on the activity of the rumen bacteria. In some instances sodium sulphate was added to the control sample to ensure that any inhibition of synthesis attributed to the metal being tested was not caused simply by an increase in the concentration of salts in the rumen liquid.

Copper Three experiments were carried out with copper, the results being recorded in Table 38. In the first experiment the rumen liquid was centrifuged for

4 minutes at 2,000 r.p.m. (800 x gravity) before adding the copper sulphate and incubating. Under these conditions with all concentrations of copper between 1 in 20,000 and 1 in 100,000 protein synthesis was reduced to negligible amounts compared with the control. In the second experiment, the preliminary centrifuging was less severe so that the liquid contained more vegetable particles, and under these circumstances 1 part of copper in 10,000 of liquid caused a marked inhibition of synthesis whereas 1 part in 100,000 had no measurable effect. In the third experiment, the rumen liquid was not centrifuged and so was very rich in vegetable matter. In this test a larger range of copper concentrations was used, and it was found that the inhibitory effect became marked between concentrations of 1 in 100,000 and 1 in 80,000. It will be noted that as the concentration of copper increased to between 1 in 40,000 and 1 in 20,000, protein breakdown predominated. This type of change from protein synthesis to breakdown as the concentration of an inhibitor increases is very similar to the effect noted by Pearson and Smith (1943, 3) when various concentrations of sodium fluoride were added to rumen liquid.

It is evident from the first two experiments (Table 38) that the presence of vegetable particles can reduce the inhibitory effect of the copper. Some of it may perhaps be bound in some way by the vegetable matter so that the effective concentration is reduced.

Samples from these copper experiments, preserved

in formalin, were examined microscopically by Mr.Frank Baker who confirmed the findings obtained by chemical analysis. Where protein synthesis was depressed bacterial counts and iodine reaction (polysaccharide formation) were correspondingly diminished.

It is of interest to compare these results with the results from feeding trials. Liebscher (1937) found that when copper was fed as copper sulphate on beet tops to milking cows at a level of 0.47 g. per 100 kg. liveweight, representing 2.13 g./1,000 lb. liveweight daily, no effect on milk production was detected. When, however, 2.86 g. per 1000 lb. liveweight was fed, a slight decrease was noted. Becker, Neal, Arnold and Rusoff (1938) on the other hand found that 1.5g./1,000 lb. liveweight daily was harmless when fed to cattle, but when the dose was raised to 2.0g. toxic effects resulted. Assuming that the rumen holds approximately 20 gallons of ingesta a dose of 2 g. copper would represent addition of copper to the rumen ingesta of approximately 1 part in 45,000. Since the in vitro experiments have shown that copper at this concentration can have a deleterious effect on microbial activity, a dose of 2g. if given all at one time might well be harmful. On the other hand if the does were given in two portions separated by an interval of several hours, or if the vegetable matter in the rumen was particularly effective in overcoming the toxicity of the copper, ill-effects with 2g. per day might not be detectable. This might explain why Becker et al. found a dose of 2g. toxic

TABLE 39

The effect of cobalt, molybdenum, iron and lead on the amount of protein synthesis obtained during the incubation of rumen liquid with 1% maltose

Expt. No.	Substance added	Concentration of the metal in the rumen liquid	Synthesis (+) or hydrolysis (-) (mg.N/100g. rumen liquid)
1	Control	-	+11.9
	Na as Na_2SO_4	1 in 1,000	+12.6
	Cobalt as acetate	(1 in 100,000	+ 8.8
		(1 in 10,000	+ 3.4
		(1 in 1,000	+ 0.6
2	Control	-	+11.6
	K as KOH	1 in 600	+11.3
	Mo as potassium molybdate	(1 in 100,000	+ 8.9
		(1 in 10,000	+ 8.6
		(1 in 1,000	+ 5.3
		(1 in 500	+ 1.0
		(1 in 250	0.0
3	Control	-	+ 6.4
	Fe as $FeCl_3$	(1 in 10,000	+ 6.0
		(1 in 1,000	+ 0.8
		(1 in 500	- 1.6
		(1 in 250	+ 2.2
	Pb as acetate	(1 in 100,000	+ 5.9
		(1 in 10,000	+ 3.8
		(1 in 1,000	+ 4.8
		(1 in 500	+ 4.5
		(1 in 250	- 0.8
4	Control	-	+10.1
	Fe as $FeCl_3$	(1 in 100,000	+11.5
		(1 in 10,000	+11.6
		(1 in 1,000	- 1.0
	Pb as acetate	(1 in 100,000	+10.3
		(1 in 10,000	+ 5.8
		(1 in 1,000	+ 7.7

whereas a slightly larger dose administered by Liebscher proved harmless.

Cobalt Cobalt was tested in much the same way as copper. It was added as the acetate to the lightly centrifuged rumen liquid. The results shown in Table 39 indicate that the addition of as little as 1 part in 100,000 had a slight affect on synthesis. With 1 part in 10,000 synthesis was very much depressed, and was almost completely inhibited when the concentration was raised to 1 part in 1,000. When it is considered that only a few milligrams of cobalt need be fed to sheep suffering from coast disease in Australia to effect recovery, it seems unlikely that the equivalent of 1 part in 100,000 would ever be added to the rumen ingesta in practice. Becker et al. (1938) did, however, feed as much as 0.5 g. per 1,000 lb. liveweight daily to cattle over a period of 5 months and noted no ill-effects. Keener, Percival, Morrow and Ellis (1947) also fed 0.5 g./1000 lb. liveweight to young dairy cattle without any ill-effects. Once more assuming the rumen capacity to be 20 gallons, a dose of 0.5 g. of cobalt would raise the concentration in the rumen by only 1 part in 180,000 which is probably within the safety limit.

Molybdenum Under Experiment 2, in Table 39, the results are shown for tests made with potassium molybdate. In order to make a solution of molybdate which would be of a convenient concentration to add to the rumen liquid

a little KOH had to be used. KOH was, therefore, added in the same concentration to one of the controls. At a concentration of 1 in 1,000 the molybdenum had a pronounced inhibitory effect. At 1 in 500 it was very small and at 1 in 250 synthesis was entirely inhibited. Since the inhibition at a concentration of 1 in 10,000 was not very marked, it seems unlikely that the scouring usually associated with "teart" pastures containing 1 part molybdenum per 10,000 dry matter results from depressed bacterial activity in the rumen. In a recent paper McGowan and Brian (1947) suggested that molybdenum may lessen the bacteriostatic action of catechols by forming catechol-molybdate complexes in the intestine and thus cause diarrhoea, and certainly catechols have been detected in grass and rumen contents and their ability to form complexes with molybdates has been confirmed by Blaschko (1947).

Iron The results for two tests with iron are shown in Table 39 (experiments 3 and 4). In the first test the results were a little anomalous with the higher concentrations, possibly due to iron at these higher levels interfering in the precipitation of protein when the N.P.N. was being estimated. But from both tests it may be concluded that concentrations up to 1 in 10,000 had no inhibitory effect whereas with 1 in 1,000 bacterial activity as measured by protein synthesis was greatly reduced. The possibility of the rumen bacteria ever being affected by iron under normal conditions is very slight.

Lead It will be seen from the second and third experiments in Table 39 that lead appeared to have a much smaller effect on the amount of synthesis than would have been expected until the concentration was as high as 1 in 250. This was very surprising since lead usually has an inhibiting effect on enzyme reactions. It may be that in the rumen liquid which is rich in carbon dioxide, lead carbonate or a complex of lead carbonate was formed in such a way that the lead was removed from solution and so prevented from exerting any inhibiting effect.

Summary and Conclusions

The effect of the metals, copper, cobalt, molybdenum, iron and lead, on protein synthesis by rumen bacteria has been studied *in vitro*. The results show clearly that copper has the most toxic effect, but the amount required to be ingested by the bovine for an inhibitory effect to be produced would be above that normally administered in treatment of deficiency diseases, and probably much greater than would be ingested from pastures treated with heavy top dressings of copper.

With cobalt, the findings were similar in that at least 2 g. would be required to be ingested at one time before any effect on the rumen flora of cattle would be observed.

Molybdenum was much less toxic than copper and cobalt. The results warranted the conclusion that scouring on "teart" pastures is almost certainly not associated with depressed bacterial activity in the rumen.

Part VI. Experiments with "Coated" Urea

Introduction

It is a well established fact that when urea is fed to ruminants under the most favourable conditions which have so far been devised, it is not utilized so efficiently as its nitrogen equivalent of protein. Thus in experiments with sheep Harris and Mitchell (1941, 1) found that under conditions in which 161 mg. casein nitrogen were required per kg. bodyweight to obtain nitrogen equilibrium, the amount of urea nitrogen required was 202 mg. The efficiency of the utilization of urea nitrogen was, therefore, about 80% of that of casein. In lactation studies with cows Owen, Smith and Wright (1943) concluded that the efficiency of utilization of urea nitrogen averaged 75% of that of blood meal nitrogen with values ranging from 53 to 88% for individual animals. There may be several reasons for the less efficient utilization of urea nitrogen, but one of the most important is probably to be found in the fact shown recently by McDonald (1948) that ammonia is readily absorbed from the rumen, for this means that much of the urea which is rapidly converted to ammonia in the rumen (Lenkeit and Becker, 1938; Pearson and Smith 1943, 2) must be absorbed before it can be utilized by the bacteria. Moreover, the ammonia will not only leave the rumen by absorption but also by passage from the rumen to the other stomachs and intestine. Some of this

ammonia will no doubt return to the rumen in the saliva, but inevitably a portion of it will be lost in the urine.

These facts led to the suggestion that urea might be more efficiently utilized if its rate of solution in the rumen liquid could be retarded in some way, and at the suggestion of Imperial Chemical Industries Ltd., the possible advantage of using samples of urea coated with starch was investigated. On theoretical grounds, such a product might be of value, for if urea were to dissolve from it reasonably slowly, a relatively constant level of N.P.N. might be maintained for the use of the bacteria, and at the same time the starch would be present as a readily available source of energy.

Several samples of coated urea were supplied to the Hannah Institute by Imperial Chemical Industries Ltd., and a few simple experiments were made to determine the rate at which urea was extracted from them by rumen liquid. The rate of extraction from feedingstuff cubes containing urea was also studied. A description of these experiments is given in the present section.

Experimental

The effect of coating on both the rate at which urea dissolved and at which it was converted to ammonia was investigated. The method adopted for estimating the solubility of urea was simply to determine the increase in the concentration of N.P.N. in the rumen liquid to which a known quantity of the coated sample had been added. Uncentrifuged rumen liquid was used. The

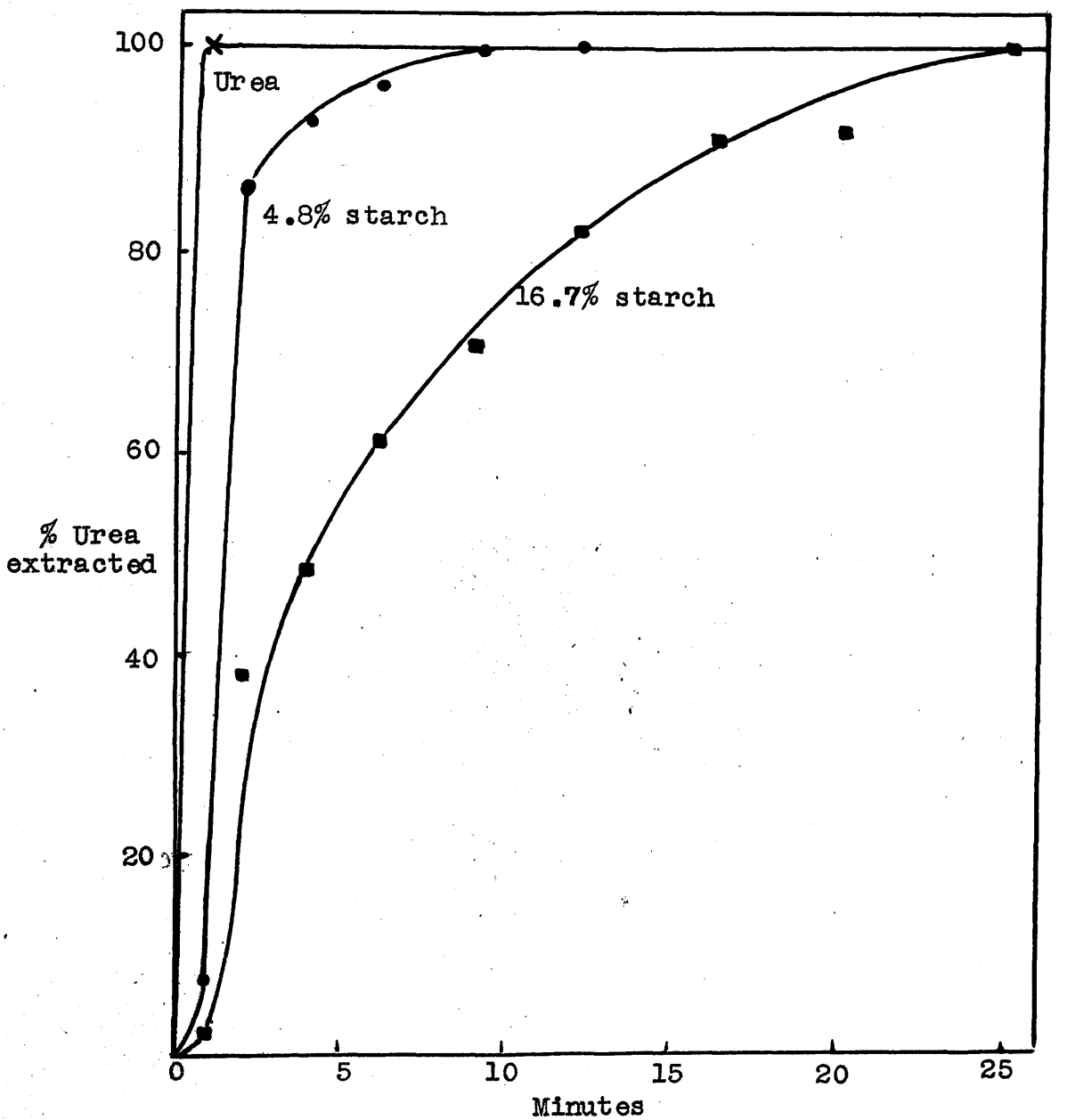


Figure 8. The rate of extraction of urea from preparations containing 4.8% and 16.7% starch, compared with the solubility of untreated urea in the same rumen liquid.

TABLE 40

The rate of extraction of urea from various preparations by rumen liquid

Expt.	Description of coated product	Amount of nitrogen extracted, expressed as a percentage of the total extractable nitrogen after the following number of minutes								
		1	2	4	6	9	12	16	20	25
1	Urea alone	100								
	Urea with 4.8% starch	7.9	86.0	92.0	96.4	99.1	100			
	Urea " 16.7% "	1.5	38.1	48.5	61.0	70.2	81.5	90.8	90.9	99.8
2	Urea with 16.7% starch	26.5	42.3	58.4	66.4	80.5	92.4	100		
	Urea " 25.9% "	43.1	57.6	74.2	82.9	89.9	94.1	95.7	97.3	100
3	Urea with 16.7% starch	17.4	34.4	51.1	65.0	78.3	87.7	95.3	100	
	Urea " 33.3% "	32.9	46.0	65.7	84.4	91.0	95.8	100		
	Urea " 41.2% "	37.0	54.4	69.1	80.5	90.5	96.2	100		
4	Urea with 33.3% starch (powder)	-	91.8	98.0	98.9	99.7	100			
	Urea " 33.3% " (particles)	31.0	39.5	60.2	70.1	80.9	93.6	96.7	100	
5	Urea with 16.7% starch	40.6	60.4	69.8	79.0	85.8	94.5	98.9	99.4	
	Urea " 3.2% stearic acid	89.6	94.7	97.7	98.1	97.9	98.7	97.2	99.9	
	Urea " 1% cellulose acetate	93.8	95.3	96.7	95.9	97.4	98.5	100.3		

mixture was stirred mechanically at 39°C and samples of the liquid were withdrawn before adding the substance under test and at intervals thereafter. Approximately 10 ml. were withdrawn at a time and immediately poured through a small piece of "clover Leaf" filter pad to remove any of the undissolved coated urea preparation. 5 ml. of the liquid were then precipitated with trichloroacetic acid and the N.P.N. and NH_3-N content of the protein-free filtrate determined.

Samples of coated urea The first experiment was carried out with samples of coated urea containing 4.8 and 16.7% starch, pure urea being used as a control. It is clear from the results of this experiment, shown in Table 40 and in Figure 8, that the starch coatings retarded solution of the urea, the rate with the 16.7% sample being much less than that with the 4.8% sample. It was natural to conclude from this that the proportion of starch present in the product was an important factor in controlling the rate of solution. Further coated samples were, therefore, prepared by Imperial Chemical Industries Ltd., containing 25.9, 33.3 and 41.2% starch, but when these products were tested together with the 16.7% sample, (Table 40 and Figure 9), it was found that the rate of solution for the 16.7% sample varied greatly from one experiment to another, and that the urea in the 33.3 and 41.2% samples dissolved at similar rates, and actually a little more quickly than the urea of the 16.7% sample.

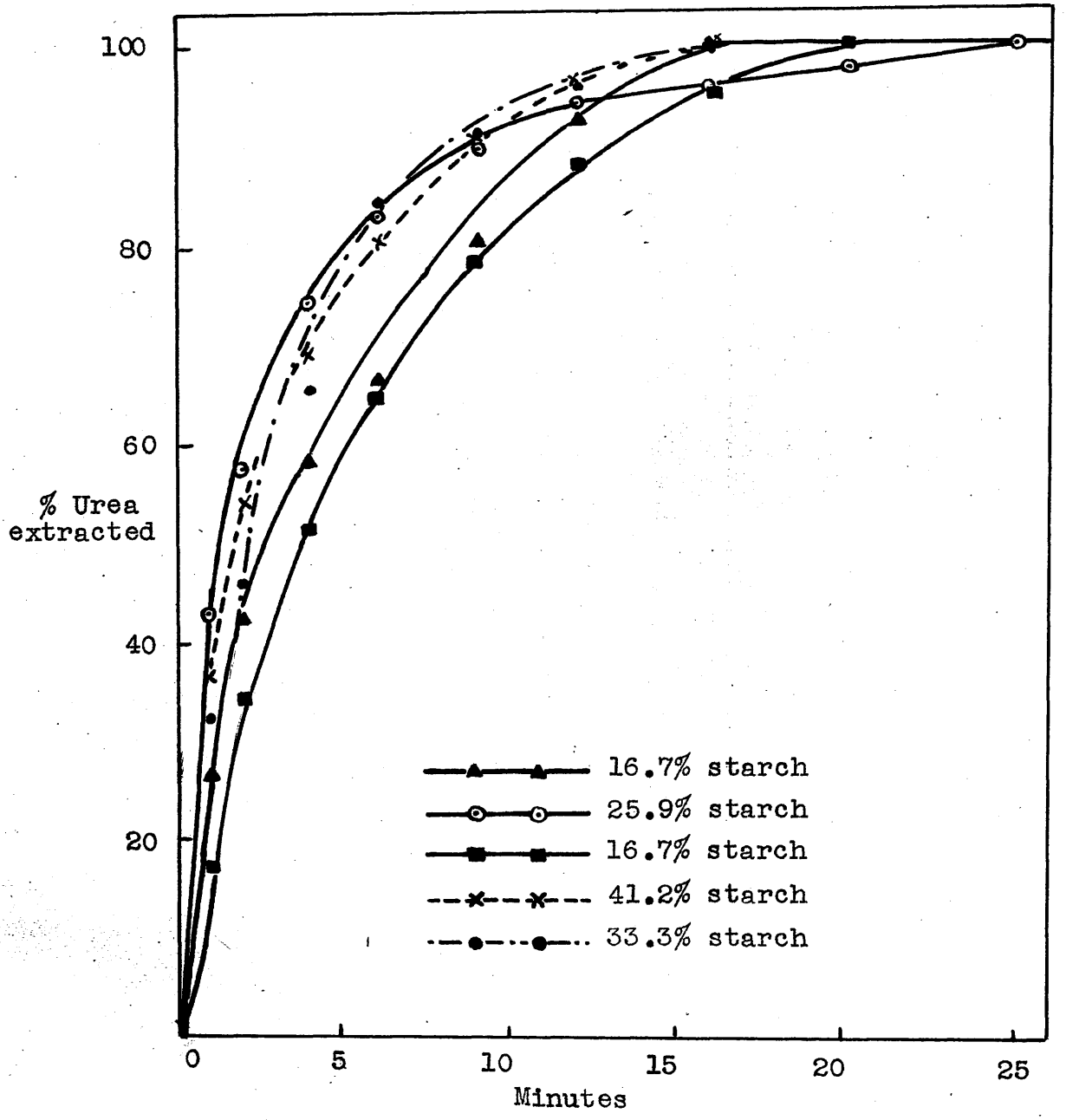


Figure 9. The rate of extraction of urea from various starch coated compounds by rumen liquid.

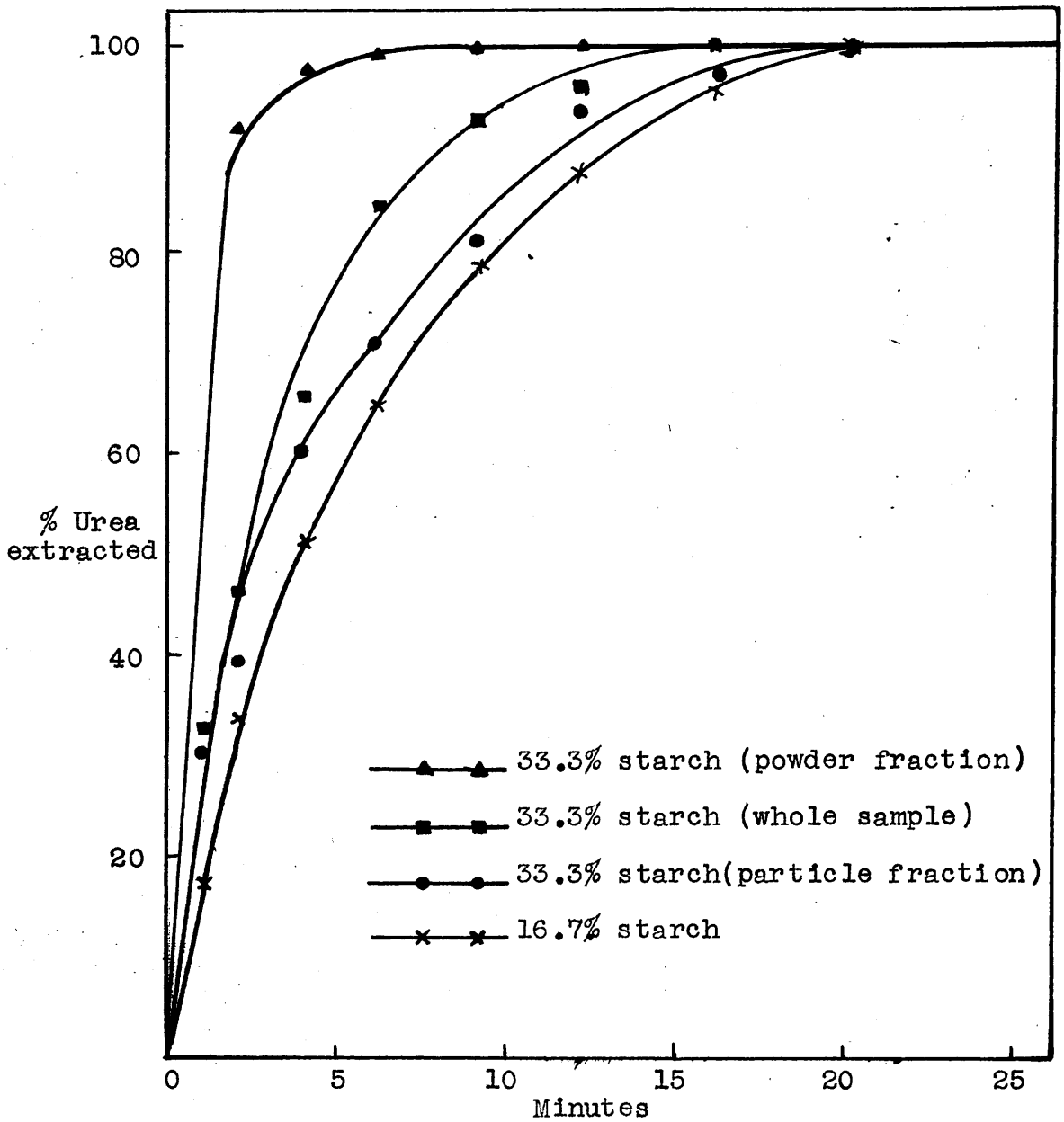


Figure 10. The effect of particle size on the rate of extraction of urea from a starch-coated urea preparation.

It appeared, therefore, that the proportion of starch present was certainly not the only factor affecting the rate of solution. Another possible factor was the size of the particles of the coated urea preparation. The 16.7% sample was observed to consist of small, medium and very large particles and it seemed probable that the different results obtained in the various tests with this sample were due to the fact that small particles predominated in the sample used in one test and larger particles in another. To obtain further information on this point, the 33.3% preparation was separated by seiving it into "powder" and "particle" fractions, and making tests with both. It is clear from the results shown in Table 40 (Experiment 4) and in Figure 10, that the rate of solution from the powder was much more rapid than from the fraction consisting of larger particles, and yet analysis showed that the two fractions contained the same amount of starch.

Since the rate of solubility depended so much on particle size, and since even at the lowest rate of solution all the urea was dissolved in 20 minutes, it did not appear that coating urea with starch would have any advantage in practice. Two further tests were made, however, with a sample of urea coated with 3.2% stearic acid and with a sample coated with 1% cellulose acetate. The results recorded for Experiment 5 in Table 40 and in Figure 11 show that these products gave a higher rate of solution than the 16.7% starch sample. They were, therefore, concluded to be of no value as a means of

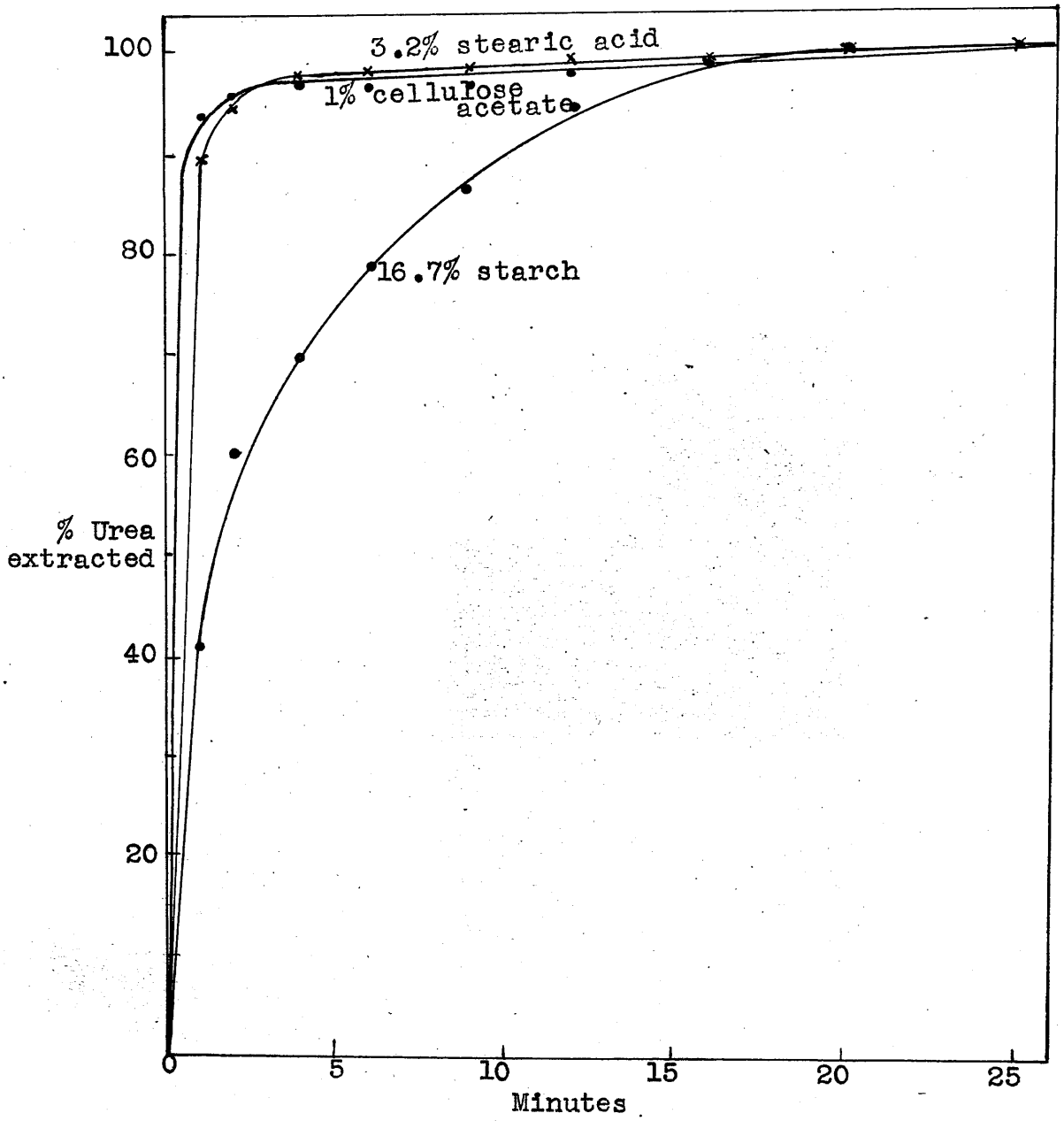


Figure 11. The rate of extraction of urea from preparations coated with starch, stearic acid and cellulose acetate.

TABLE 41

The conversion to ammonia of the urea, extracted from coated preparations, by rumen liquid

Experiment No.	Description of coated product	Amount of NH_3-N in the ingesta in mg./100g. liquid after the following number of minutes									
		0	1	2	4	6	9	12	16	20	25
1	Urea	10.0	16.7	15.9	19.8	19.8	27.8	29.6	34.3	36.8	
	Urea with 16.7% starch	12.1	14.1	16.9	18.0	18.8	27.9	27.4	37.7	39.7	45.6
	Urea " 4.8% "	8.2	8.6	14.9	19.8	23.7	24.8	28.1	36.6	43.1	
2	Urea with 16.7% starch	17.3	21.3	25.2	26.4	31.9	36.9	42.2	42.8	49.7	55.9
	Urea " 3.2% stearic acid	12.2	23.3	29.1	30.0	29.0	32.9	43.8	47.7	49.7	58.1
	Urea " 1% cellulose acetate	18.1	22.1	27.0	30.9	31.9	35.9	37.8	44.7	49.7	55.6

increasing the efficiency with which urea would be utilized in practice.

The rate at which ammonia was formed from urea when several of the samples were stirred with rumen liquid at 39°C was also determined. The results in Table 41 show that after 2 minutes or so, the amount of ammonia formed was as great from the coated samples as from pure urea itself.

Urea in feedingstuff cubes Undoubtedly one of the best methods by which urea can be fed to cattle is to incorporate it in feedingstuff cubes. The urea can be dissolved in molasses and the molasses introduced into the feedingstuff mixtures during the cubing process. This is very readily done on a manufacturing scale, and cubes made in this way have been found by Snow, Smith and Wright (1945) to keep well under normal storage conditions. In view of the experiments which have just been described, it was of interest to determine the rate at which urea was dissolved from cubes of this type by rumen liquid. Two tests were made. In the first, some of the cubes were roughly crumbled to represent what might happen when they were chewed, and the rate at which urea was extracted from the product was compared for a sample of the same rumen liquid with the rate of extraction from the sample of coated urea containing 16.7% starch. In the second test the cubes were crumbled and divided into two fractions, one consisting of lumps and the other of powder, and the rates of solution from these two fractions compared. The results are shown

TABLE 42

The rate of extraction of urea from a starch-coated preparation and from feedingstuff cubes containing urea

Experiment No.	Description of product	Amount of nitrogen extracted expressed as a percentage of the total extractable nitrogen after the following number of minutes								
		1	2	4	6	9	12	16	20	25
1	Urea with 16.7% starch	19.8	31.4	38.2	50.3	87.4	91.8	94.2	96.3	100
	Cubes containing urea	15.1	23.4	35.9	55.4	68.5	75.6	86.5	93.2	100
2	Cubes containing urea (lumps)	6.3	22.7	33.9	55.8	74.7	91.5	96.8	100	
	Cubes " " (powdered)	21.7	48.8	66.2	92.6	97.5	100			

TABLE 43

The N.P.N. and NH_3-N contents of the rumen liquid before and after an animal had eaten rations consisting of hay and a concentrate mixture containing 3% urea

Time of sampling	N.P.N.	NH_3-N	Synthesis during incubation for 4 hr.
	(mg. N/100 g. rumen liquid)		
Fasting	5.2	1.9	1.7
Time after the meal (hr.)			
1	22.2	18.9	6.9
2	15.4	11.8	
3	7.3	5.2	
4	4.0	1.8	1.2
6	3.5	0.8	
8	3.9	1.6	

in Table 42 and Figure 12.

It will be seen that by incorporating urea in feedingstuffs cubes and crumbling the cubes, its solubility was delayed to about the same extent as by coating with 16.7% starch. Once again particle size was important, the rate of solution from the powdered product being considerably greater than from the more lumpy material. It will be noted, too, that even from the feedingstuff sample consisting of relatively large lumps, all the urea was dissolved in 20 minutes.

The rapidity with which ammonia disappears from the bovine rumen when urea is fed was shown in the present work by results such as those recorded in Table 43. Before a feed containing urea, the levels of N.P.N. and NH_3-N in the rumen liquid in this particular experiment were 5.2 and 1.9 mg. per 100 g. One hour after the feed they had greatly increased to values of 22.2 and 18.9 respectively. Four hours after the feed, however, they had returned again to the initial levels. Samples of the liquid were tested before the feed and one hour and 4 hours after for their "synthesising power", and it was clear that protein synthesis could not in itself have accounted for the large and rapid decrease in the levels of N.P.N, and NH_3-N which occurred in the first few hours after feeding. Much of the N.P.N. must have been absorbed into the blood stream or passed further along the alimentary tract. The fact that in this experiment the increase in the level of N.P.N. was accompanied by an increase in synthesising

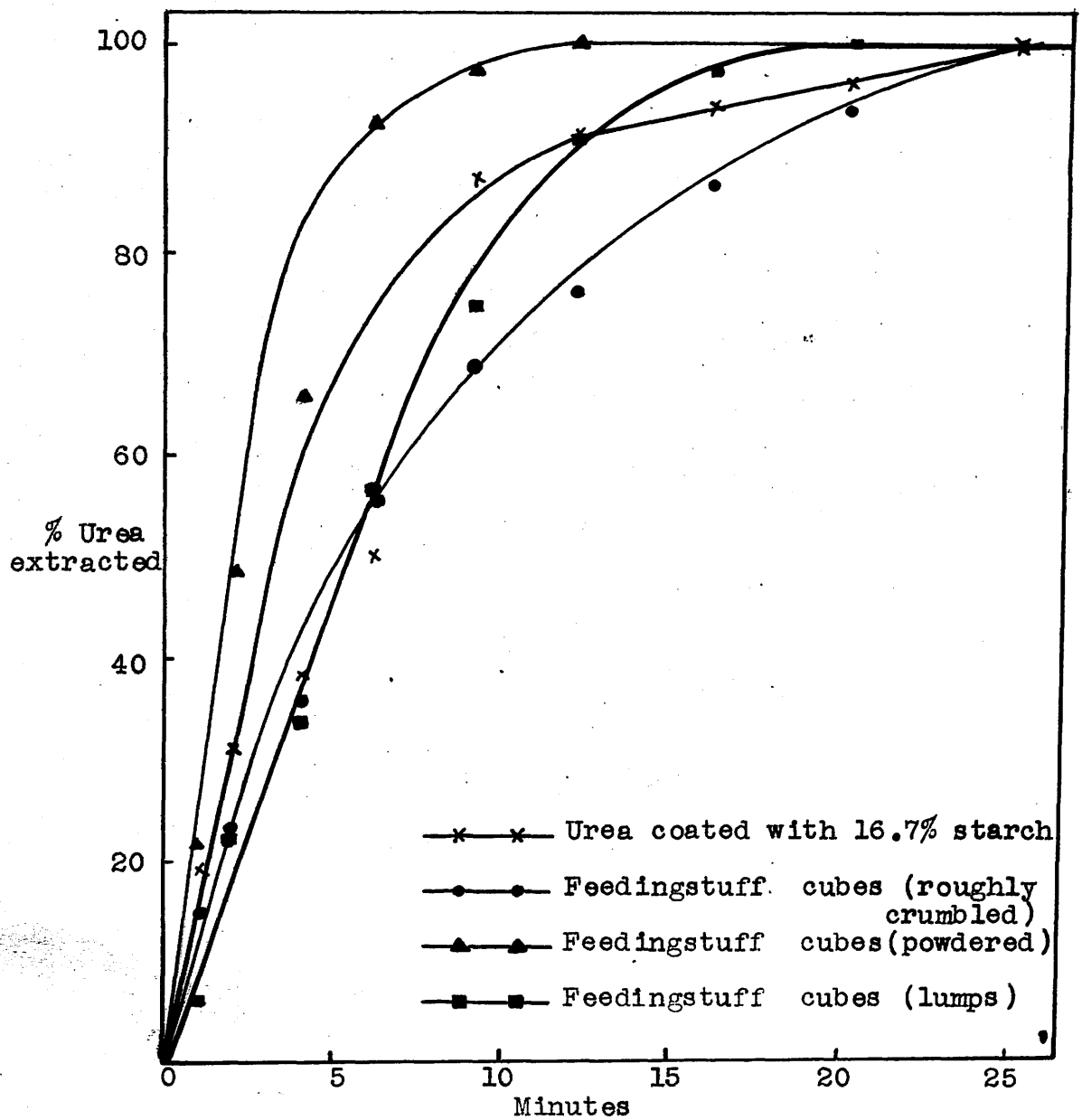


Figure 12. The rate of extraction of urea from feedingstuff cubes and from urea coated with 16.7% starch.

power suggests that the optimum N.P.N. level was probably not attained. It follows from these observations that the discovery of some method of feeding N.P.N. in a form from which ammonia would be liberated slowly between feeds might well increase the efficiency with which the N.P.N. would be utilized. The experiments just described indicate that coating urea is not likely to help in this direction and that coating has no advantage over the incorporation of urea in feedingstuff cubes.

Summary and Conclusions

It has been suggested that utilization of N.P.N. by ruminants might be more efficient if the N.P.N. was liberated into the rumen liquid slowly throughout the whole 24 hours of the day instead of just immediately after feeding.

Several samples of urea coated with various proportions of starch and with stearic acid and cellulose acetate were supplied by Imperial Chemical Industries Ltd., and a study made of the rate at which rumen liquid extracted urea from these preparations and converted it to ammonia. Similar tests were made with feedingstuff cubes containing urea.

The rate at which urea was extracted from the feedingstuff cubes was much the same as with the coated products. Even with the slowest rate of solution all the urea was dissolved completely after 20 minutes. A method by which urea can be liberated slowly into the rumen liquid from feedingstuff mixtures containing urea has still to be devised.

REFERENCES

Archibald, J.G. (1943). Mass.agric.Exp.Sta.Bull.No.406.

Armsby, H.P. (1911). U.S.Dept.Agric.Bur.Animal Indust. Bull. No.139.

Association of Official Agricultural Chemists, (1945). Official and Tentative Methods of Analysis (6th edition). Washington, D.C.

Baker, F., (1943). Ann.appl.Biol., 30, 230.

Baker, F. and Harriss, S.T., (1947-48). Nutr.Abstr.Rev. 17, 3.

Barker, S.B., and Summerson, W.H. (1941). J.biol.Chem., 138, 535.

Bartlett, S. and Blaxter, K.L. (1947). J. agric.Sci., 37, 32.

Bartlett, S. and Cotton, A.G. (1938). J.Dairy Res., 9, 263.

Becker, E.R. (1929). Proc.Nat.Acad.Sci.Wash., 15, 435.

Becker, R.B., Neal, W.M., Arnold, P.T.D. and Rusoff, L.L. (1938). Rep.Fla.agric.Exp.Sta. 62-63.

Benesch, R. (1941). Nature, Lond., 147, 531.

Blaschko, H. (1947). Nature, Lond., 159, 373.

Boivin, A., Vendrely, R. and Tulasne, R. (1947). Bull.Acad.Med. 131, 39.

Braude, R. (1942). J.Inst.Brew. 39, 206.

Breirem, K. (1947). Proc.Nutrit.Soc. 5, 330.

Brouwer, E. (1947). PProc.Nutrit.Soc. 5, 350.

Dougherty, R.W. (1941). J.Amer.vet.med.Assoc., 99, 110.

Elsden, S.R. (1945-46). J.exp.Biol. 22, 51.

Fiske, C.H. and Subbarow, Y. (1925). J.biol.Chem., 66, 375.

Goss, H. (1942-43). Nutr.Abstr.Rev., 12, 531.

Guilleman, M. and Larson, W.P. (1922). J. infect. Dis. 31, 355.

Hagemann, O. (1891). Landwirtsch Jahrb. 20, 264.

Hale, E.B., Duncan, C.W. and Huffman, C.F. (1940). J. Dairy Sci., 23, 953.

Hammond, J. (1928). Vet. Record, 9, 343. (new series)

Harris, L.E. and Mitchell, H.H. (1941, 1). J. Nutrit. 22, 167.

Harris, L.E. and Mitchell, H.H. (1941, 2). J. Nutrit. 22, 183.

Harris, L.E., Work, S.H., and Henke, L.A. (1943). J. Anim. Sci., 2, 328.

Hart, E.B., Bohstedt, G., Deobald, H.G. and Wegner, M.I. (1939). J. Dairy Sci., 22, 785.

Hastings, E.G. (1944). Bact. Rev., 8, 235.

Henry, K.M., Kon, S.K. and Watson, M.B. (1937). Milk and Nutrition, Part 1. p.37. Nat. Inst.Res. Dairying, Reading.

Johnson, B.C., Hamilton, T.S., Mitchell, H.H. and Robinson, W.B.(1942). J. Anim. Sci., 1, 236.

Johnson, B.C., Hamilton, T.S., Robinson, W.B. and Garey, J.C. (1944). J. Anim. Sci., 3, 287.

Keener, H.A., Percival, G.P., Morrow, K.S. and Ellis, G.H. (1947). J. Dairy Sci., 30, 557.

Kleiber, M., Cole, H.H. and Mead, S.W. (1943). J. Dairy Sci., 25, 929.

Kohler, W. (1940). Archiv.Mikrobiol. 11, 432.

Kon, S.K. and Porter, J.W.G. (1947-48). Nutr.Abstr.Rev. 17, 31.

Krebs, K. (1937). Biederm. Zbl. (B) Tierernährung, 9, 394.

Lenkeit, W. and Becker, M. (1938). Z.Tierernahr.Futtermittelk, 1, 97.

Liebscher, W. (1937). Proc. 11th Int.Dairy Congr. 1, 130.

McAnally, R.A. (1943). Onderstepoort J.vet.Sci., 18, 131.

McAnally, R.A. and Phillipson, A.T. (1944). Biol. Rev., 19, 41.

McCrudden, F.H. (1911-12). J.biol.Chem., 10, 187.

McDonald, I.W. (1948). Biochem. J., 42, XXXIII.

McGowan, J.C. and Brian, P.W. (1947). Nature, Lond., 159, 373.

McNaught, M.L. and Smith, J.A.B. (1947-48). Nutr.Abstr.Rev. 17, 18.

Martin, W.McK. and Green, J.R. (1933). J.industr.Engng.Chem. (anal.ed.) 5, 114.

Mills, R.C., Booth, A.N., Bohstedt, G. and Hart, E.B. (1942). J. Dairy Sci., 25, 925.

Mills, R.C., Lardinois, C.C., Rupel, I.W. and Hart, E.B. (1944). J. Dairy Sci., 27, 571.

Mitchell, H.H. (1924,1). J. biol. Chem. 58, 873.

Mitchell, H.H. (1924,2). J. biol. Chem. 58, 905.

Mitchell, H.H. and Carman, G.G. (1926). J. biol. Chem. 68, 183.

Mitchell, H.H. and Hamilton, T.S. (1929). The Biochemistry of the Amino Acids, Chemical Catalog Co., New York.

Monroe, C.F. and Perkins, A.E. (1939). J. Dairy Sci., 22, 983.

Mowry, H.A. and Becker, E.R. (1930). Iowa St.Coll.J.Sci., 5, 35.

Müller, M. (1906). Pflugers Arch., 112, 245.

Myburgh, S.J. and Quin, J.I. (1943). Onderstepoort J. vet. Sci., 18, 119.

Olsen, T.M. (1940). J. Dairy Sci., 23, 343.

Olsen, T.M. (1942). J. Dairy Sci., 25, 684.

Owen, E.C. (1941). J. Dairy Res., 12, 213.

Owen, E.C. (1947). J. Dairy Res., 15, 142.

Owen, E.C., Smith, J.A.B. and Wright, N.C. (1943). Biochem. J., 37, 44.

Pearson, R.M. and Smith, J.A.B. (1943, 1). Biochem. J., 37, 142.

Pearson, R.M. and Smith, J.A.B. (1943, 2). Biochem. J., 37, 148.

Pearson, R.M. and Smith, J.A.B. (1943, 3). Biochem. J., 37, 153.

Phillipson, A.T. (1942). J. exp. Biol., 19, 186.

Phillipson, A.T. (1947-48). Nutr.Abstr.Rev., 17, 12.

Phillipson, A.T. and McAnally, R.A. (1942). J. exp. Biol., 19, 199.

Rupel, I.W., Bohstedt, G. and Hart, E.B. (1943). J. Dairy Sci., 26, 647.

Schalk, A.F. and Amadon, R.S. (1928). N. Dak. agric. Exp.Sta.Bull. No.216.

Smith, J.A.B. and Baker, F. (1944). Biochem. J., 38, 496.

Snow, D., Smith, J.A.B. and Wright, N.C. (1945). J. agric. Sci., 35, 65.

Steensberg, V. (1947). Proc.Nutrit.Soc., 5, 322.

Usuelli, F. and Fiorini, P. (1938). Boll.Soc.ital. Biol.sper. 13, 11.

Van der Wath, J.G. (1942). Thesis - University of Pretoria.

Van der Wath, J.G. and Myburgh, S.J. (1941). Onderstepoort J. vet. Sci., 17, 61.

Washburn, L.E. and Brody, S. (1937). Mo. agric. Exp. Sta. Bull. No.263.

Watts, P.S. (1948). Vet. Record, 60, 4.

Wegner, M.I., Booth, A.N., Bohstedt, G. and Hart, E.B. (1940). J.Dairy Sci., 23, 1123.

Wegner, M.I., Booth, A.N., Bohstedt, G. and Hart, E.B. (1941,1). J.Dairy Sci., 24, 51.

Wegner, M.I., Booth, A.N., Bohstedt,G. and Hart, E.B. (1941,2). J.Dairy Sci., 24, 835.

Whitehouse, K., Zarow, A. and Shay, H. (1945). J.Ass.off.agric.Chem.Wash., 28, 147.

Willett, E.L., Henke, L.A. and Maruyama, C. (1946). J.Dairy Sci., 29, 629.

Zuntz, N. (1891). Pflugers Arch., 49, 483.