The Fermentation of Cellulose *in vitro* by Organisms from the Rumen of Sheep

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The suggestion that micro-organisms are responsible for the decomposition of cellulose in the alimentary canal (Wildt, 1874; Zuntz, 1879; Duclaux, 1882) arose from the proof (Henneberg & Stohmann, 1860-4) that it is, in fact, cellulose which disappears during the passage of crude fibre through the animal (Haubner, 1855). Tappeiner (1882, 1883, 1884, 1888), who was the first to investigate the fate of cellulose, found that paper pulp and cotton wool, when incubated under anaerobic conditions with the fluid contents of the rumen, dissolved slowly with the evolution of methane and carbon dioxide and the formation of fatty acids. He ascribed little nutritional value to the fatty acids, however, and suggested that the fermentation of cellulose was important only because it allowed the digestive enzymes access to the contents of the plant cells (Tappeiner, 1884). This view was not accepted (Henneberg & Stohmann, 1885); the fact that only traces of fatty acids appear in the excreta (Wilsing, 1885; von Knieriem, 1885) implies at least that they are absorbed and oxidized. There was already positive evidence of the nutritional value of cellulose (von Knieriem, 1885; Lehmann, 1889). Apart from the investigations of Markoff (1911, 1913) which added little to the information provided by Tappeiner (1884) this aspect of ruminant digestion was abandoned in a state of considerable confusion.

Tappeiner (1888) distilled the medium from somewhat prolonged *in vitro* fermentation of cellulose by rumen contents, and from the combining capacity and composition of the silver salts of the mixed volatile fatty acids in the distillate, claimed that acetic and butyric acids were the main products of fermentation. Quite apart from the possibility that his cultures had been qualitatively affected by the environment in which the fermentations were carried out, the chemical methods upon which the claim was based were not capable of distinguishing between mixtures of these two acids and any combination of the lower homologues. Examination of his data reveals no sound reason for the conclusion that only the 2-C and 4-C acids were among the products. On the occasion when he examined a series of fractions collected during progressive distillation Tappeiner (1884, p. 91) concluded, 'Zweifelhaft bleibt das Vorkommen von Propionsäure'. His data clearly indicated the presence of considerable quantities of propionic acid.

Markoff's cultures also were probably subject to environmental alteration, and apparently he was unaware that the

higher homologues distil first from a mixture of fatty acids in aqueous solution, and that an analytical technique based on distillation constants had been published by Duclaux (1874, 1895, 1900). He concluded without sound reason that the average molecular weight of the fatty-acid products of his fermentations of cellulose was the same as that of butyric acid (Markoff, 1913, p. 36). There was thus no real justification for the assumptions which led Honcamp (1909, 1927) to derive empirical equations for the degradation of cellulose in the alimentary canal.

Before the experiments described here were started* we were aware that considerable quantities of propionic acid appeared in the rumen during fermentation, but acetic acid usually predominated and sometimes accumulated in greater excess over the propionic acid than would be expected even if the pentosans of the fodder were fermented preferentially (Seillière, 1910) to acetic acid. This fact was difficult to reconcile with our observations on the apparent overall usefulness to the animal of the products of rumen fermentation. It appeared from the work of Lusk and his colleagues (Ringer & Lusk, 1910; Lusk, 1921; Milhorat & Deuel, 1926-7) that, in the dog at least, acetic acid was rapidly and wastefully oxidized without entering the carbohydrate cycle; and it was obvious from energy transactions that the ruminant derives more useful energy from its fodder than would be implied if the greater proportion of the carbohydrates were converted to acetic acid and subsequently disposed of without entering into the productive channels of metabolism.

Although these *in vitro* fermentations were conducted under conditions as similar to those obtaining in the rumen as were technically feasible, a considerable preponderance of propionic acid accumulated. This finding was in more reasonable agreement with the results of some calorimeter experiments on sheep carried out in this laboratory, which we hope to publish shortly, but the (acetic)/(propionic) ratio was so different from that found in the rumen contents as to lead to a suspicion that the community of micro-organisms responsible for the fermentations *in vitro* had been so altered that the course of degradation had been changed materially from that which normally prevails in the paunch.

The confirmation by Barcroft and his colleagues of Trautmann's (1933) observation that absorption may take place directly through the rumen wall (Barcroft, McAnally & Phillipson, 1944a), and their convincing demonstration

* The experiments were carried out in part at the Biochemical Laboratory, Cambridge, during 1937 when the author was the guest of the late Sir Frederick Gowland Hopkins, and in part at the author's laboratory, Adelaide, in 1938.

that considerable quantities of fatty acids find their way into the blood stream draining the rumen (Barcroft *et al.* 1944 b, c), provided the clue which led to a more complete understanding of ruminant digestion and to a better appreciation of the energy transactions involved in the utilization of the fermentation products. Further experiments have shown that when the contents of the rumen are acid, as they normally are during active digestion, propionic acid is absorbed preferentially to acetic acid (Danielli, Hitchcock, Marshall & Phillipson, 1945; Gray, 1947). The relative rates of diffusion through the rumen wall are such as may normally reverse the ratio of acetic/propionic acids in the contents of the rumen. Conclusions based only on analyses of rumen contents may thus be misleading. The necessity for further knowledge of the products of *in vitro* fermentations conducted in an environment similar to that of the rumen is obvious.

The environment in the rumen

The environment in the rumen is the main factor which regulates the community of micro-organisms responsible for carbohydrate degradation. Outstanding features of this environment are:

(a) *Salivary secretion.* A relatively large quantity of dilute alkaline saliva is secreted continuously by the parotid glands.* Besides its buffering capacity and its ability to maintain a high CO_2 tension in the medium where the fermentation proceeds, the parotid saliva carries to the micro-organisms a supply of phosphate and simple nitrogenous compounds, which is in very considerable excess of the quantity usually available from the fodder.

(b) *The reaction of the rumen contents.* When in a quiescent state, 24 hr. after ingestion of food, the rumen contents are about neutral. In samples from

* Colin (1886) estimated that approximately 60 l. of parotid saliva are secreted each day by the ox. Scheunert & Trautmann (1921) concluded that the sheep secretes approximately 4 l. in 24 hr., which is probably a low estimate. In an investigation preliminary to these *in vitro* fermentation experiments, the author established fistulae in the left parotid ducts of three mature ewes and, when the secretion was passed back into the mouth at frequent intervals, collected up to 3.5 l. of saliva in 24 hr., which would imply the secretion of approximately 7 l. each day under the particular feeding conditions of the experimental animals. They were fed at noon each day with 1.2 kg. of a mixture of chaffed cereal and lucerne hays, and consumed the whole of it within 2 hr. (cf. Marston, 1939). In these studies there was evidence of reflex stimulation by the acidity of the rumen contents, and so a reasonable estimate was possible only if the secretion was collected during the whole cycle of 24 hr. Chemical analyses of the saliva confirmed Scheunert & Trautmann's (1921) estimate of the bicarbonate concentration (equivalent to 0.1–0.2 N- NaHCO_3) and indicated that both the phosphate and the urea, which became concentrated in the secretion, reflected changes in the inorganic phosphate and urea concentrations of the blood. Watson (1933), in this laboratory, had established previously that the concentration of phosphate in the blood was related to that of the mixed saliva collected on sponges inserted into the mouth.

twelve animals taken at slaughter and collected without loss of CO_2 , the reaction at 40° ranged between pH 6.40 and 7.14, with a mean of pH 6.8. The fatty acids produced during fermentation are in part neutralized by the saliva, and in part removed either by passage to lower levels of the alimentary canal or by direct diffusion through the rumen wall. When fermentation is most vigorous, during the period between 4 and 8 hr. after feeding, acid production exceeds these modifying influences; the mass then becomes distinctly acid, sometimes less than pH 5.

(c) *The oxidation-reduction condition of the liquid contents and the gas tension of the atmosphere above.* The partial pressure of oxygen is usually less than 8% in the atmosphere above the liquid contents of the quiescent rumen 24 hr. after feeding. This is augmented temporarily by the oxygen in the air swallowed with the food. It is diminished abruptly to very low levels (cf. Washburn & Brody, 1937), mainly through displacement by the CO_2 and the methane evolved when active fermentation begins soon after feeding. The liquid contents of the rumen, however, maintain a low oxidation-reduction potential and, even after the animal has fasted 3 days, the conditions are such as to allow some methane production to proceed. Normally there is a high partial pressure of CO_2 in the rumen gas at all times.

Experimental imitation of the milieu

The low partial pressure of oxygen and the high CO_2 tension, the supply of phosphate and inorganic nitrogen from the saliva, the buffering capacity of the saliva, the temperature of approximately 40°, and the residual contents of the rumen, which retain a massive inoculum of a community of micro-organisms already selected by the *milieu*, are apparently the main factors which determine the course of fermentation in the normal rumen. When subjected to these conditions, the complex carbohydrates and simple sugars are fermented very rapidly. Some observations (unpublished) on the rate of disappearance of cellulose from different levels of the intestinal tract of sheep indicated that a digestion mass of approximately 5 l. in the rumen of a sheep fermented in 8 hr. from 400 to 600 g. of mixed carbohydrates, at least half of which was cellulose, and that the rate of dissimilation at the peak of activity exceeded considerably the average over this period. The rapid rate of fermentation and of absorption of the products was reflected in the energy metabolism of the sheep: after feeding with 1 kg. of hay the metabolic rate increased within an hour to a level approximately 25% above that previous to feeding, remained there for 6–7 hr., and then receded during the ensuing 2 hr. to the lower level which was maintained until the animal was fed again 24 hr. later.

EXPERIMENTAL

Apparatus. The vessel in which the fermentations were carried out was a 3.5 l. glass pot, hermetically sealed with a metal lid clamped down on a rubber gasket (Fig. 1). A stirrer attached to a geared variable-speed motor which could at will be run very slowly, passed through a mercury-sealed bearing in the lid. Six ports, tapered to fit standard

Fig. 1. Fermentation apparatus consisting of a 3.5-l. closed jar with mercury-sealed stirrer and six ports through which subsidiary apparatus was introduced.

rubber stoppers, through which other pieces of apparatus could be introduced, were attached to the lid. All metal parts within the pot were coated with paraffin wax. The subsidiary apparatus (Fig. 2) consisted of: an inlet and an outlet tube for gas, *A*, one of which was constructed to take the delivery point of a burette through which the standard alkali could be introduced; a standardized glass electrode for pH measurements, *B*; a bright platinum electrode, *C*, for oxidation-reduction potential measurement; a saturated calomel half cell, *D*, to provide a reference potential; a closed pipette, *E*, into which a sample of the liquid contents of the pot could be drawn up around a second glass electrode which was used on occasions to check the accuracy of the glass electrode, *B*; and a toluene-mercury thermoregulator

which, through a relay, operated the heating elements of the water bath in which the pot was immersed. The transfer of heat through the pot was not sufficiently rapid to ensure very precise temperature regulation: the contents varied $\pm 1.5^\circ$ about the 40° setting.

The ingoing stream of N_2 was purified by passing over soda-asbestos and then over freshly reduced Cu maintained at approximately 600° in a silica-tube furnace. The outgoing stream, after dilution with approximately twice its volume of CO_2 -free O_2 , was dried by passing through H_2SO_4 and then over anhydrous $Mg(ClO_4)_2$ (Dehydrite) and the CO_2 absorbed with soda-asbestos (Ascarite) in two vessels placed in sequence. These were large enough to deal efficiently with the considerable volume of CO_2 evolved as a fermentation product, and from the acid decomposition of the Na_2CO_3 which was introduced during the digestion. Guard tubes containing anhydrous $Mg(ClO_4)_2$ were introduced into the chain at each point immediately after the CO_2 absorbers. The dry, CO_2 -free gas was then led through the combustion chain which is described in a later section.

Substrate, culture medium and inoculum. The cellulose used in Exps. 1 and 2 was a specimen provided by Dr David Bell and described by him as 'practically pure α -cellulose from birch wood'; in Exps. 3 and 4 it was prepared from filter paper (Munktell 1 F) which had been suspended in 17.5% (w/v) NaOH for 1 hr., then washed with dilute HCl and subsequently with water until free from chlorides. It was then dried. Both samples were reduced to fine powder (100 mesh). In this state the cellulose was more tractable during the fermentation.

The salts in the inorganic medium were the usual 'analytical reagent' grade which, being by no means free from heavy metals, would probably have carried with them sufficient of these constituents to meet the requirements of the micro-organisms. However, to ensure that the course of digestion would not be influenced by the want of these minor elements which are present always in the normal fodder of ruminants, Fe, Cu, Zn and Co were added to the medium as solutions of the chlorides in sufficient quantity to provide 2 mg. Fe, 1 mg. Cu, 2 mg. Zn, and 0.5 mg. Co/l. of the inorganic medium.

For each digestion, approximately 50 g. (vac. dry wt. 100°) of the finely divided cellulose was weighed and suspended in 2.5 l. of a solution containing the following concentrations of inorganic constituents: 0.02 M- $(NH_4)_2SO_4$, 0.02 M- KH_2PO_4 , 0.01 M- $MgSO_4$, 0.005 M- $CaCl_2$, together with the traces of heavy metals mentioned above.

The micro-organisms which were used as an inoculum were separated mechanically from the rumen contents of sheep which for some days had been fed on chaffed cereal hay. At slaughter, the paunch was tied off and the contents (3-5 l.) removed and strained through a sieve of fine gossamer while being vigorously stirred; this and all subsequent operations were conducted under an atmosphere of CO_2 . The liquid portion was freed further from gross contaminating particles by gentle centrifuging. The micro-organisms in the supernatant liquid were then separated in a Sharples centrifuge. The residual sludge was removed from the cylinder, emulsified in 0.2 M-phosphate buffer,

pH 5.5, and made up to 50 ml. The CO_2 was swept out with pure N_2 . Samples for cellulose and carbon determinations were withdrawn and the remainder (which contained between 5 and 8 g. of wet sludge) was transferred without delay to the digestion vessel, already prepared to receive it. The material consisted largely of bacteria, the main contaminants being small fragments of lignin, lignocellulose and some of the micro-fauna which normally inhabit the rumen.

The oxidation-reduction (O/R) potential of the medium during fermentation. During active digestion the potential set up on the indifferent (Pt) electrode immersed in the medium indicated an E_h at pH 7.0 poised in the vicinity of -0.380 V. The observed potentials fluctuated, sometimes rapidly, over a relatively wide range (approximately ± 30 mV.). The system responsible seemed too delicately balanced to suggest even an approach to a state of true equilibrium; the more negative potentials suggested rather

Fig. 2. Subsidiary apparatus introduced through ports in fermentation apparatus: inlet and outlet tubes for gas, etc., and glass, calomel and platinum electrodes. The thermoregulator introduced through the sixth port is not shown.

Procedure of fermentation. After introducing the cellulose, the vessel was closed, the subsidiary apparatus made ready and the contents raised to 40° . The inoculum was run in through one of the ports. The air was swept out with a rapid stream of pure N_2 passed through the inlet tube beneath the surface while the mash was being stirred rapidly. The inlet tube was then withdrawn above the surface, the flow of gas reduced to about 300 ml./hr. and the efferent stream, after dilution with O_2 , diverted to the absorption and combustion chain. Standardized 0.5 M- Na_2CO_3 was added drop by drop from the burette, with gentle stirring, until the reaction of the contents was pH 6.8. Stirring was then stopped. Active digestion usually began within an hour and became increasingly rapid. The rate was judged by the change in reaction of the contents as indicated by the glass electrode. The reaction was adjusted at frequent intervals (10–20 min.) by introducing standard Na_2CO_3 from the burette; it was kept by this means between pH 6.8 and 5.5 throughout the course of each fermentation. The stirrer was used only when adjusting the reaction, and then at a rate sufficient merely to impart a gentle swirl to the medium. Rapid stirring was found to reduce the rate of fermentation.

In this way the *milieu* of the rumen was imitated. The conditions were at least very similar to those which prevail normally. The inoculum was relatively massive, and there was a minimum of selection of micro-organisms by the mechanical processes employed to separate them from the normal rumen contents; the reaction of the medium was controlled; the conditions were anaerobic and the CO_2 tension high; and the duration of the fermentation was relatively short. It might reasonably be expected that the course of dissimilation of the cellulose under these conditions would be close to, if not identical with, that taken during normal digestion in the paunch of ruminants.

The results of four successful digestions are reported here.

the production of evanescent, highly reducing intermediates, possibly of hydrogen itself. Active fermentation of cellulose was invariably associated with such low potentials; from observations, however, it was not possible to conclude whether they represented an essential condition for the function of the organisms or whether they were merely the result of the formation of cellulose dissimilation products.

RESULTS

The products of the fermentations in vitro

Gaseous products: CO_2 and CH_4 . Throughout the course of each experimental digestion the atmosphere above the fermenting mass was swept with pure N_2 , continuously, but sufficiently slowly to ensure that the CO_2 tension was not reduced to low levels. The efferent stream, after it had been diluted with pure O_2 and freed quantitatively from H_2O and CO_2 , was led through a silica tube packed with CuO and maintained at approximately 700° , and thence through anhydrous $\text{Mg}(\text{ClO}_4)_2$ and soda-asbestos absorbers to collect the H_2O and CO_2 arising from the combustion. At the completion of a digestion period the introduction of alkali was stopped, the mass was allowed to become acid (pH 5.8–6.0) and then 5 ml. H_3PO_4 (sp. gr. 1.6) were delivered through the inlet port. The N_2 delivery tube was pushed well down beneath the surface, the stream of gas increased, and the mass agitated vigorously with the mechanical stirrer. This procedure was not prolonged further than would ensure removal of the fermentation gases without washing out any appreciable quantity of the more volatile products of digestion.

Methane was the only combustible gas evolved under these conditions of fermentation. Within the very small error of the estimation there was no evidence that the gas evolved contained any free

hydrogen: the ratios of CO_2 and H_2O arising after combustion invariably indicated a carbon/hydrogen ratio of 1:4 atoms.

The formation of methane from hydrogen has been recognized since Söhngen (1905) demonstrated the reduction of CO_2 by H_2 with enrichment cultures from soil. Its formation as a product of the CO_2 oxidation of ethanol by *Methanobacterium omelian-skii* (Barker, 1936a) or of simple fatty acids by *Methanococcus mazei* and by *Methanobacterium söhngeni* (cf. Barker, 1936b) are well established reactions. Its production from any compound containing a carbon chain, by an organism isolated by Stephenson & Stickland (1933), reveals further the variety of possible sources of methane in anaerobic fermentations with mixed cultures. The fact that free hydrogen is evolved during the dissimilation of cellulose by 'enrichment' and by 'pure' cultures, or by mixed cultures under conditions which favour thermophilic organisms but in which methane producers could not function, suggests that the bulk of the methane evolved from the rumen may be formed from hydrogen during primary fermentation of complex polysaccharides. Decomposition of other products would be a subsidiary source of methane. Both types of reaction almost certainly occur.

There is thus no reason to expect an inflexible relationship between the amount of methane produced, the amount of substrate fermented, and the CO_2 evolved in the process.

The ratios $(\text{CO}_2)/(\text{CH}_4)$ in the four fermentations under discussion varied between 3.0 and 3.8 (see Table 2). As the estimation of these constituents was precise, this variation may be attributed to differences in the course of the activity of the mixed cultures. The changes in the ratios do not correlate simply with similar changes in the ratios of the volatile fatty acids, nor with the degree of exhaustion of the substrate. The differences might thus have been due in these experiments to changes in the nature of the original inoculum. The partial pressure of CO_2 above the fermenting mass was not rigidly controlled and may at times have deviated from that which normally prevails in the rumen during active digestion. The fact that free hydrogen was not evolved indicates, however, that at least the CO_2 oxidation of hydrogen was not at any time limited by the CO_2 tension. Between 6.2 and 8.1% of the combustible energy of the cellulose digested was contained in the methane evolved.

The main products: fatty acids. The major part of the undissolved cellulose was collected on a sintered glass funnel. The organisms and fine particles of cellulose which passed the filter were then spun off in a Sharples centrifuge and the residue washed. The insoluble fractions were combined and the cellulose content determined by the method of Norman & Jenkins (1933).

The clarified filtrate and washings were adjusted with NaOH to pH 8.0 and evaporated to about 250 ml. in a still, to which was attached an efficient condenser and a close-fitting receiver immersed in a freezing mixture. Steam was then admitted to the flask and the distillation continued until an additional litre of distillate had collected in the cold receiver. The distillate (D_1) was preserved for the estimation of volatile alcohols, aldehydes, ketones, etc. The residue in the flask was concentrated to approximately 250 ml., acidified with H_3PO_4 to pH 3.0 and then steam distilled without increasing its volume, until approximately 5 l. of distillate (D_2) had collected. The residue (R_1) was preserved for the estimation of succinic acid and the semi-volatile lactic and pyruvic acids. The distillate (D_2) was returned to the still, rendered alkaline, and the volume reduced again to approximately 250 ml. The distillate (D_3) from this, after applying qualitative tests for alcohols, ketones, etc. was discarded. The residue in the flask was acidified again to pH 3.0, and steam distilled until 5 l. of distillate (D_4) had been collected. The residue from this (R_2) was combined with (R_1) and treated as described in the section on intermediates. The distillate (D_4) was exactly neutralized with a known quantity of $\text{Ba}(\text{OH})_2$, computed from careful titration of a sample, and then cautiously reduced to dryness and dried finally in a vacuum desiccator. From the weight of the Ba salts and the Ba content the mean combining capacity of the mixed salts was computed. This provided a useful means of evaluating the overall accuracy of the subsequent analyses.

The Ba salts were then taken up in water, decomposed with the calculated quantity of H_2SO_4 , and the mixed fatty acids extracted with pure ether in a continuous extraction apparatus. After removal of the ether they were fractionated in an apparatus designed specially for quantitative separation of mixtures of this sort. The column employed was an unpacked spiral glass tube, approximately 10 m. in length, sealed together with a totally enclosed copper reflector into a glass vacuum jacket, the whole being encircled externally with an electrically heated casing. This latter and the external insulation allowed precise adjustment of the thermal gradient. The rate of entry of vapour into the column was determined by a thermoregulator situated at its head, and means were provided for controlling the quantity of liquid taken off. When the apparatus was adjusted critically a sharp separation of the constituents was ensured with a minimum of retention in the fractionating column. The efficiency of the column was equivalent to 24 theoretical plates.

The following fractions were collected: F_1 , up to 101°, which contained water and formic acid; F_2 , from 101 to 119°, which contained the acetic acid; F_3 , from 119 to 142°, which contained the propionic acid; F_4 , from 142 to 164°, which contained butyric acid. Transition between the fractions was abrupt. Formic acid was determined in F_1 by reduction of HgO (Osburn, Wood & Werkman, 1933) and butyric acid in F_4 and in the residues by oxidation to acetaldehyde (Kline, 1934). The composition of fractions F_3 and F_4 was established further by the refractive index and by the combining capacity and the composition of the anhydrous barium salts. The presence of higher homologues in the residuum was doubtful: if they did occur they were in very small quantities.

The results from complete analyses of the products of four digestions are set out in Table 1. The

minor products were not determined in digest 1, but in subsequent experiments these were sought for and estimated quantitatively. They were unimportant in the overall balance sheets of the transactions involved, and the estimations added but little to the understanding of the intermediary stages of the dissimilation.

Elsden (1945), employing partition chromatographic means (Martin & Synge, 1941; Smith, 1942; Ramsey & Patterson, 1945; Elsdén, 1946) for the separation of the fatty acids, found in the rumen contents a preponderance of acetic over propionic acid, but he recognized the fact that propionic acid predominated over acetic acid when carbohydrates

Table 1. *The products of fermentation of cellulose*

(mmol. = millimols/'mol' (162 g.) of cellulose digested.)

Digest	Formic acid	Acetic acid	Propionic acid	Butyric acid	Methane	CO ₂	Cellulose digested (g.)	Duration of digestion (hr.)
1	0.08 g. 12 mmol.	5.3 g. 600 mmol.	11.6 g. 1060 mmol.	0.27 g. 21 mmol.	0.6 g. 250 mmol.	5.4 g. 830 mmol.	24	26
2	0.17 g. 34 mmol.	4.0 g. 610 mmol.	8.5 g. 1050 mmol.	0.3 g. 31 mmol.	0.46 g. 260 mmol.	3.9 g. 810 mmol.	17.8	24
3	0.11 g. 11 mmol.	7.8 g. 620 mmol.	14.4 g. 925 mmol.	1.68 g. 91 mmol.	0.66 g. 195 mmol.	6.9 g. 750 mmol.	34	36
4	0.21 g. 44 mmol.	3.1 g. 500 mmol.	6.4 g. 840 mmol.	1.2 g. 130 mmol.	0.39 g. 236 mmol.	3.2 g. 710 mmol.	16.7	48

The most striking of the analytical findings was the proportion of the main products, propionic acid and acetic acid, and their relationship to the cellulose digested. This relative constancy in the four digests could hardly be fortuitous: it indicates a strong tendency for the main course of dissimilation to proceed through similar if not identical channels. Either the populations of micro-organisms which constituted the inocula were of extraordinarily similar composition, or the selective forces of the environment brought about a relatively stable associative growth. It is probable that the latter were mainly responsible. The inocula were separated from the rumen contents of sheep all of which had been fed similarly for a considerable period prior to slaughter. Each population of micro-organisms thus had ample opportunity to become stabilized.

Table 2. *Molecular ratios of main constituents*

Digest	(Propionic)/(Acetic)	(CO ₂)/(CH ₄)
1	1.8	3.3
2	1.7	3.1
3	1.5	3.8
4	1.7	3.0

The ratios of propionic acid and acetic acid produced in these digests (Table 2) were so similar to those found by Van Niel (1928) to result from the fermentation of lactic acid by certain strains of *Bact. acidii propionici*, as to suggest that organisms of the genus *Propionibacterium* were concerned in their production. Formic acid and butyric acid were present in all digests. The amounts were small and their proportions much more variable. It would appear that these are the products of subsidiary and relatively unstable channels of dissimilation.

were fermented under anaerobic conditions in inorganic media inoculated with liquor withdrawn from the paunch. The findings from the *in vitro* fermentations under discussion here are in substantial agreement except for the wide variability of the acetic/propionic ratios found by Elsdén. There is, however, no real reason to conclude that the normal course of carbohydrate dissimilation in the rumen varies to the extent implied by Elsdén's observations. He made little serious attempt to duplicate the *milieu* of the rumen; the inocula employed were small and the duration of the fermentations prolonged. The possibility that the course of the dissimilation might be altered under such circumstances was greater than when the conditions of the rumen were imitated and the environment controlled throughout.

Intermediate products. In mixed cultures of this sort, the metabolic end products of the organisms responsible for primary fission of the cellulose would be exposed to further dissimilation. In addition, each soluble intermediate would be subject to the potential competitive action of other organisms, always attendant and ready with a wide diversity of enzyme systems to take advantage of suitable substrates. Intermediary products under such circumstances would be more than usually evanescent and the end products themselves to some degree transient, for the extent of accumulation would be a measure of relative, rather than of complete, stability in this environment.

An exhaustive study of the intermediate compounds may thus not greatly illuminate the processes involved in these mixed fermentations. A search was made, however, for products of subsidiary channels of dissimilation, which might accumulate

in sufficient concentration to be of some nutritional importance to the ruminant. The proportions of propionic and acetic acids in digest 1 suggested that the main course of the dissimilation might proceed through lactic acid (see above). For this reason lactic acid was estimated in digests 2, 3 and 4. The following data from digest 2 are typical of the findings.

Reducing sugars were absent. The distillate (D_1) contained 13 mg. acetaldehyde. Residues (R_1) and (R_2) were combined, reduced in volume, then acidified and exhausted with ether by continuous extraction. The small quantity of extractive, after freeing from ether, was taken up in water and the succinic acid, the aldehydes and ketones, and the semi-volatile lactic and pyruvic acids were estimated. Oxidation of a sample by a modification (Friedemann, Cotonio & Shaffer, 1927) of the von Fürth & Charnass (1910) procedure produced a quantity of acetaldehyde which would imply the presence of approximately 30 mg. lactic acid in the whole digest. The total sulphite-combining capacity of the extractive was of an order which would correspond to less than the equivalent of 6 mg. pyruvic acid, and this would be the upper limit of concentration. As more than 70% of this was destroyed on heating with alkali the concentration of pyruvic acid must have been very small. The quantity was insufficient to allow it to be isolated as the 2:5-dinitrophenylosazone. About 7 mg. succinic acid were identified and about half this amount was isolated. A small quantity of an unidentified ketone or aldehyde was present.

It would appear that lactic acid rather than pyruvic acid tended to accumulate in these digests. This, however, is no proof that lactic acid was a more important intermediate than pyruvic acid in the dissimilation: the reverse might well be true. The prevention of the reduction of lactate by the addition of fluoride would have provided useful evidence on this point, but at the time when the experiments were conducted neither Barker & Lipmann (1944) nor Chaix-Audemard (1940) had published their interesting studies. Attempts were made, which however proved abortive, to accumulate intermediate complexes in the digest by applying Neuberg's *Abfangsverfahren*. Sulphite, when added to an actively fermenting culture apparently acted as a hydrogen acceptor, and altered the course of dissimilation. Hydrogen sulphide was evolved; nitrogenous compounds, which were either introduced in the original inoculum or had been elaborated since, underwent putrefactive changes; in contrast to the light colour and faint sweet odour of the contents of the normal rumen, or of the *in vitro* fermentations under discussion, the mass darkened and stank of indole and mercaptans. Intermediates accumulated by this means would have been of doubtful significance, and so the task of isolating them was set aside.

The minor products identified were not in sufficient quantity to be a nutritionally important energy

source for the animal, and they were too small to be considered in the computations of the energy and carbon balance sheets.

The carbon and combustible energy balance sheets

In the four digests that were analyzed in detail, between 84 and 93% of the carbon of the cellulose transformed was recovered in the main products of digestion (see Table 3). There was no evidence which would suggest that either the intermediary complexes or the products from subsidiary channels of dissimilation ever accumulated to an extent which would influence materially the carbon balance sheets. The losses incurred in the chemical isolations were not great, and the small experimental errors entailed in the estimation of the relative proportions of the fatty acids would have comparatively little influence on the assessment of the overall carbon content of these main products. It is not unreasonable, then, to conclude that by far the greater part of the carbon deficit could be accounted for by the carbon bound in the cells of the micro-organisms during their proliferation and growth.

An attempt was made to assess this directly. The main bulk of the undissolved cellulose of digest 3 was separated from the finer particles and micro-organisms by shaking vigorously and straining through fine gossamer. The gross residue was washed in this way several times. The washings were passed through the supercentrifuge after the original filtrate. Cellulose was estimated in the residues which did not pass the sieve and in the sludge from the supercentrifuge by the method of Norman & Jenkins (1933), and the total carbon in the latter was determined by the wet-digestion method of Backlin (1930). The difference between the total carbon content of these residues and the carbon of the cellulose in them was taken as carbon bound in micro-organisms, corrections being applied for the carbon introduced in the original inoculum. Approximately 75% of the carbon deficit in the soluble products was accounted for in this way. It was at best an approximate estimate. Sampling difficulties were encountered, and only a partial recovery of micro-organisms was realized.

The proportion of the carbon of the digested cellulose which was present in the fermentation products was greater when the period of digestion was not prolonged. This suggests that as digestion proceeded, conditions became more favourable for the multiplication of organisms other than those which brought about the primary cleavage of the cellulose; the extent of proliferation of these latter might be expected to bear a more direct relationship to the amount of cellulose digested while this substrate was present in large excess.

The carbon and combustible energy-balance sheets (Tables 3 and 4) indicate that the cellulose fermentation products contained 6% less combustible energy than that of the cellulose fermented. This deficit is an approximate estimate of the free

Table 3. *Carbon contents of the constituents of four digests analyzed in detail*

Digest	Formic acid carbon (g.)	Acetic acid carbon (g.)	Propionic acid carbon (g.)	Butyric acid carbon (g.)	Methane carbon (g.)	Carbon dioxide carbon (g.)	Cellulose digested carbon (g.)	Carbon recovered in products (%)
1	0.02	2.1	5.6	0.15	0.45	1.5	10.7	92
2	0.04	1.6	4.1	0.16	0.34	1.1	7.9	93
3	0.03	3.1	7.0	0.91	0.49	1.9	15.1	89
4	0.06	1.25	3.1	0.65	0.29	0.85	7.4	84

Table 4. *Combustible energy contents of the constituents of four digests analyzed in detail*

Digest	Formic acid (kg.cal.)	Acetic acid (kg.cal.)	Propionic acid (kg.cal.)	Butyric acid (kg.cal.)	Methane (kg.cal.)	Carbon dioxide (kg.cal.)	Cellulose digested (kg.cal.)	% of kg.cal. in products
1	0.1	18.5	57.5	1.6	7.9	—	100	86
2	0.2	14	42	1.8	6.0	—	74	87
3	0.1	27	71	10	8.7	—	140	83
4	0.3	10.8	32	7.1	5.1	—	70	79

energy in the conversion of cellulose to these products, as entropy changes in transformations of this sort are sufficiently small to be within the experimental errors entailed in the chemical estimations on which the computations were based. This energy would be available for the metabolic processes of the micro-organisms. As the relationships between the carbon and combustible-energy contents of cellulose and of the organisms which proliferate during these fermentations are approximately the same,* the amount of energy dissipated as heat in the exothermic metabolic processes of the micro-organisms would be practically identical with the free energy of the dissimilation. The energy balance sheet of the fermentation would thus be as set out in Table 5.

Table 5. *The energy balance sheet of the dissimilation*

Digest	Combustible energy of cellulose fermented		
	In soluble products and methane (%)	In micro-organisms (%)	Dissipated as heat (%)
1	86	8	6
2	87	7	6
3	83	11	6
4	79	16	5

The fact that the temperature of the rumen contents during active fermentation of hay is above body temperature was pointed out by Krzywanek (1929).

* The combustible energy in bacteria of this type is close to 9.2 kg.cal./g. carbon, the carbon content being about 50 % of their dry weight and the combustible energy between 4.6 and 4.7 kg.cal./g. dry weight (Rubner, 1904). In cellulose it is 9.4 kg.cal./g. carbon, the carbon content being 44.4 % and the combustible energy 4.18 kg.cal./g. The former would thus be approximately 98 % of the latter.

DISCUSSION

Rumen fermentation and the energy metabolism of the ruminant

The associative growth of organisms in the rumen

It seems likely that the course of dissimilation of cellulose in the rumen is determined primarily by the *milieu*, for it is highly probable that any of the vast variety of micro-organisms which inhabit the soil might find their way into the paunch. Those able to flourish anaerobically on the intermediary and end products of the primary fission of complex carbohydrates would form, with the organisms responsible for this first cleavage, a more or less stable population whose composition would be determined by the mutually beneficial relationships between the strains able to take their place in this community.

It might be expected that under the influence of the selective forces of the environment, the process of dissimilation by an associative growth of this sort would tend strongly to assume a pattern, which in many respects would be analogous to the orderly series of dehydrogenations which take place when the simpler carbohydrates are degraded under aerobic conditions in the animal organism.

Viewed in this light, it would not be remarkable if the nature and proportions of the products of fermentation in the rumen were similar, irrespective of the variations in composition of the substrate which obtain under natural feeding conditions. The simple fatty acids represent a relatively refractory stage of carbohydrate dissimilation in the environment of the paunch and, for this reason, accumulate. They are removed from the rumen too rapidly for secondary changes to assume any but an unimportant role. If, however, the *in vitro* digests are left exhausted of their substrate, further anaerobic degradation of these temporary 'end products' takes place. The

final stage would probably be the complete conversion to carbon dioxide and methane found by Hoppe-Seyler (1886) when cellulose was fermented for 4 years with cultures from mud.

The influence of starch and other supplements on the energy transactions in the rumen

If diets containing a high proportion of starch are fed, the rate of proliferation of the microflora in the rumen is limited usually by the nitrogen supply, for, when additional nitrogen is provided as ammonium salts or as simple amides, a large proportion of it is converted to protein through the rapid proliferation of micro-organisms. A considerable part of the combustible energy of the starch may, under these circumstances, escape in part the losses inevitably associated with fermentative degradation. The thermodynamic costs entailed in the production of these organisms are not known, and so the overall effects, which microbial syntheses in the presence of starch have on the supply of energy to the animal, cannot at present be assessed.

When the substrate is composed essentially of the cellulose, celluloses and polyuronides, etc. of the natural fodder of ruminants, proliferation of the microflora is not influenced so materially by the nitrogen supply, and supplements of simple nitrogenous compounds are utilized poorly if at all by this population. The combustible energy in the bacteria, which pass from the rumen of sheep on this fodder, may be considerably less than the amount estimated from the *in vitro* fermentations discussed above, for *in vivo* the products of fermentation are removed from the rumen and so proliferation at their expense is minimized. In the overall energy economy of an animal on its natural fodder this course in the energy transactions of rumen digestion, although an important adjunct, is a subsidiary one; there is little doubt that the fatty acids arising from the dissimilation of carbohydrates in the paunch provide for the ruminant its main source of energy.

The usefulness of the fermentation products as sources of energy to the animal

The high degree of efficiency, with which the ruminant is able to derive energy from the more complex carbohydrates in its fodder, is offset by the losses associated with the fermentation of starch and of the simpler sugars. In other animals these latter suffer no appreciable loss of combustible energy when they are hydrolyzed by the enzymes of the alimentary canal. They are absorbed as simple sugars before the ingesta pass to the caecum, where micro-organisms ferment in part the more refractory carbohydrate complexes which are unchanged by the digestive enzymes.

The energy represented by the heat dissipated in the metabolic processes of the micro-organisms

responsible for the dissimilation in the rumen would be of no benefit to an animal in a warm environment: under cold conditions, however, when the heat radiated by the animal exceeds that evolved by the normal processes of its metabolism, this fraction would spare its equivalent of the combustible energy of nutrients which otherwise would be oxidized in order to maintain body temperature. The combustible energy of that part of the carbohydrates which becomes converted to micro-organisms is apparently well utilized by the animal, for there is ample evidence that micro-organisms which proliferate in the paunch are well digested and are absorbed when they pass to lower levels of the tract. A considerable proportion of this fraction, however, would be represented as protein, and so would be subject to the usual losses associated with protein metabolism.

The products of the rumen fermentation of carbohydrates differ widely in their usefulness to the animal. Methane is expelled and so its combustible energy will be a total loss. Butyric acid might be expected to be utilized efficiently in the fat cycle. The whole of the combustible energy of the propionic acid is probably capable of supporting the processes involved in the maintenance, growth and production of the animal; propionic acid is readily convertible to glucose (Ringer, 1912; Deuel, Butts, Hallman & Cutler, 1935-6) and so would enter the carbohydrate cycle. Acetic acid, however, is by no means so adaptable in metabolism. While there is a great body of evidence which indicates that in the animal it is not convertible to carbohydrate precursors (Ringer & Lusk, 1910; Ringer, 1913; Deuel & Milhorat, 1928; Ponsford & Smedley-MacLean, 1932; Stöhr, 1933; Deuel *et al.* 1935-6; Cross & Holmes, 1937), it is not improbable from more recent evidence that the fat and carbohydrate cycles may be linked through 2-C compounds. The ease and versatility of carbon dioxide fixation, however, renders difficult the assessment of energy costs incurred during the transfer of acetic acid into productive channels. As carbon dioxide labelled with isotopic carbon finds its way into glycogen (Solomon, Vennesland, Klemperer, Buchanan & Hastings, 1941; Buchanan, Hastings & Nesbitt, 1942; Vennesland, Solomon, Buchanan & Hastings, 1942) the interpretation of the mechanism, through which the carbon isotope from C¹³-carboxyl-tagged acetate is transferred to glycogen, is complicated, and as yet there is no general agreement as to the course it takes (Buchanan *et al.* 1943; Lorber, Lifson & Wood, 1945).

If acetic acid is rapidly oxidized and its combustible energy dissipated as heat without entering into the productive channels of metabolism, its combustible energy must be classed with the heat evolved in the exothermic metabolic processes of the

micro-organisms responsible for the dissimilation of carbohydrate in the rumen, for it would serve only to spare other foodstuffs when the animal is in a cold environment. If acetic acid enters the carbohydrate cycle without appreciable loss, its combustible energy should be classed with that of propionic and butyric acids.

In the former case the products of the *in vitro* fermentations of cellulose would have for the animal a productive value between 67 and 72 % of the combustible energy of the cellulose fermented (i.e. 2.8–3.0 kg.cal./g. cellulose); in the latter, if acetic acid follows a course similar to that of propionic acid or butyric acid and enters without loss into either the carbohydrate or fat cycle, the productive value of the fermentation products would be between 85 and 88 % of the combustible energy of the cellulose (i.e. 3.6–3.7 kg.cal./g. cellulose).

The problem of the comparative usefulness of acetic and propionic acids in ruminant nutrition has been investigated through the direct approach of animal calorimetry. Experiments to determine the capacity of acetate and propionate to provide energy, to spare tissue destruction in the fasting sheep and to promote a positive energy balance when added to maintenance rations will be discussed elsewhere.

SUMMARY

1. Under environmental conditions similar to those in the normal rumen, cellulose, when fermented *in vitro* by associative growths of micro-organisms separated from actively fermenting rumen contents, yielded acetic acid, propionic acid, carbon dioxide and methane as main products, together with smaller quantities of formic acid and butyric acid, and traces of acetaldehyde, pyruvic acid and lactic acid.

Propionic acid predominated among the dissimilation products; the molecular ratio of propionic to acetic in four digests ranged from 1.5 to 1.8. The molecular ratios of carbon dioxide to methane ranged from 3.0 to 3.8.

2. Depending on the duration of the fermentation the amount of combustible energy in the micro-organisms which proliferated ranged between 7 and 16 % of that of the cellulose fermented. The cost of production and maintenance of these organisms, estimated from the carbon and energy balance sheets, was approximately 6 % of the combustible energy of the cellulose fermented. As the relationship between the carbon and combustible-energy contents of the micro-organisms and that of the cellulose is approximately the same (9.4 kg.cal./g. C), the energy dissipated as heat during fermentation was close to the energy deficit of the products of fermentation, viz. 6 % of the combustible energy of the cellulose fermented.

3. The energy metabolism of the ruminant is discussed in light of these findings, and it is concluded that the useful energy of the dissimilation products would be between 85 and 88 % of the combustible energy of the cellulose (i.e. between 3.6 and 3.7 kg.cal./g. cellulose) if the acetic acid enters without loss into productive channels of metabolism, and between 67 and 72 % of the combustible energy of the cellulose (i.e. between 2.8 and 3.0 kg.cal./g. cellulose) if the acetic acid is oxidized and wastefully dissipated. These figures would apply to the ruminant in a warm environment; under cold conditions both heat evolved during the dissimilation and the combustible energy of the acetic acid, irrespective of whether it is otherwise useful to the animal, would be available to spare other nutrients which would be oxidized to maintain body temperature.

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The Estimation of Suramin in Plasma

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The colorimetric estimation of suramin (Antrypol, Bayer 205) by conversion of the hydrolytic fission products to an azo dye has been described by several investigators. Dangerfield, Gaunt & Wormall (1938) have summarized the earlier work, and have described in detail a method in which the drug is subjected to acid hydrolysis; the fragments are then diazotized and coupled with methyl- α -naphthylamine. Spinks (1948) has used alkaline hydrolysis and *N*- β -sulphatoethyl-*m*-toluidine as a dye-stuffs end component. Balaban & King (1927), in their investigation of the affinity of suramin and other complex ureides for cellulose, devised a

roughly quantitative method in which the concentration was determined from the turbidity produced by the addition of a basic protein such as clupeine sulphate; for this method the authors claimed a sensitivity of 1 in 100,000.

In this investigation the possibility of a direct, colorimetric estimation by a dye-laking procedure has been explored by examining the behaviour of a range of basic dyes in the presence of the drug. Certain styrylquinoline dyes have been found to exhibit a marked colour change under these conditions, and 2-*p*-dimethylaminostyryl-6-acetamidoquinoline methochloride has been selected as the