The Biochemistry of Rumen Protozoa

1. CARBOHYDRATE FERMENTATION BY *DASYTRICHA* AND *ISOTRICHA*

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Of the various Protozoa found in the rumen of sheep and cattle, the holotrichous ciliates are represented by two genera: *Isotricha*, containing *I. intestinalis* and *I. prostoma*, and *Dasytricha*, containing only *D. ruminantium*. The two genera are closely related, the most obvious morphological difference being the greater size of the two *Isotricha* species. The biochemical properties of these organisms were first investigated by Oxford (1951) and Masson & Oxford (1951), who found that adding glucose to strained sheep-rumen liquor caused the holotrichs to sink to the bottom of the vessel as a dense white layer by virtue of the considerable amount of amylopectin synthesized and deposited inside the cells. Such suspensions, washed almost free of bacteria, were used by Sugden & Oxford (1952) in cultural studies, and by Heald, Oxford & Sugden (1952) and Heald & Oxford (1953) in manometric studies. The last-named authors found that mixed suspensions of the three species, in the presence of bactericidal concentrations of streptomycin, carried out a vigorous anaerobic fermentation of a number of sugars and polysaccharides. The chief products of the fermentation of glucose were acetic, butyric and lactic acid, carbon dioxide, hydrogen and amylopectin.

The first suggestion that the two genera might differ biochemically was made by Sudgen & Oxford (1952), who observed that when rice-starch grains were added to a mixed suspension of the holotrichs, only the isotrichs were seen to swallow the grains. The isotrichs were thus enabled to survive, whereas the *D. ruminantium* died. Gutierrez (1955) was the first to examine suspensions of either genus apart from the other. He separated organisms of the two genera from a mixture by taking advantage of differences in the sedimentation characteristics. By using these suspensions, it was shown that *D. ruminantium* was able to ferment cellobiose, but the isotrichs could not. Mould & Thomas (1957) observed that the amylases obtained from the two genera differed in electrophoretic mobility.

It is of obvious interest to investigate further these differences between the genera of rumen holotrichs. A method of obtaining pure suspensions of either genus is required which is more convenient than that of Gutierrez (1955), and which also eliminates the possibility of incomplete separation of the two types of organism.

The experiments in this Institute on removing fauna from sheep rumens (Eadie & Oxford, 1957) were designed partly toward this end. These workers were able to remove all holotrich Protozoa from the rumen and, since transfer of these organisms is solely from mouth to mouth (Becker & Hsiung, 1929), the treated sheep remained in that condition indefinitely if kept in isolation. It was further found possible to re-establish in such a sheep a culture of either *D. ruminantium* or mixed *Isotrichia* species by adding a suspension of the organisms, which had been individually separated under a dissecting microscope from a mixture of holotrichs obtained from another sheep. The population increased to a suitable level in 3–5 weeks. When the usual procedure for obtaining holotrich suspensions was applied to rumen liquor from such sheep, good yields of either *D. ruminantium* or *I. prostoma* plus *I. intestinalis* were obtained. Experiments with such suspensions are described here. This work has been already briefly reported (Howard, 1957*a*).

MATERIALS AND METHODS

Sheep containing a single genus of holotrich. The removal of fauna from, and establishment of *D. ruminantium* in, a single sheep has already been described (Eadie & Oxford, 1957). A mixture of *I. intestinalis* and *I. prostoma* was similarly established in the rumen of another fistulated sheep. Both sheep were initially fed on hay only, but as the animals began to lose weight and appetite, and the protozoal population fell, the diet was supplemented by a small proportion of grass cubes during the course of these experiments.

Preparation of protozoal suspensions. Samples of 1 l. of rumen liquor were collected in the morning about 2 hr. after feeding, and treated as described by Eadie & Oxford (1955). Unless stated otherwise, glucose was used to cause settling of the Protozoa, and the buffer solution of Sugden & Oxford (1952) was used for washing. By frequently changing the buffer, the isotrichs could be kept alive for over 48 hr., by which time the endogenous fermentation rate had fallen to a low level. The *D. ruminantium* suspensions, however, were invariably dead or dying after being kept overnight, and it was therefore necessary to use the

D. ruminantium suspensions on the day of collection, allowing only a few hours' starvation. The washed suspensions of Protozoa were sufficiently free from bacteria to make the use of antibiotics unnecessary (Heald & Oxford, 1953).

Substrates. Sugars were either Kerfoot's Biochemical Reagents or AnalaR; glucosides were supplied by L. Light and Co. Rice starch was a gift of Reckitt and Colman Ltd., Hull. Holotrich-starch grains were prepared as described in the next paper (Howard, 1959). Holotrich starch was defatted by boiling under reflux with two successive portions of 85% (v/v) methanol in water. Precipitated starch was made by dissolving the grains in hot water and precipitating with ethanol.

Manometric experiments. The same buffer solution was used in the Warburg cups as for washing the Protozoa; it includes 0·1% of $NaHCO_3$. The gas phase was $N_2 + CO_2$ (95:5), freed from traces of O_2 by bubbling through Hungate's (1950) chromous sulphate reagent. The side arms contained 0·2 ml. of buffer solution in which was dissolved 10μmoles of sugar or glucoside or, for di- and trisaccharides, an amount yielding 10μmoles of fermentable sugar on hydrolysis. An exception was aesculin which, because of its relative insolubility, could be used at the rate of only 4μmoles/cup. Protozoal suspension (1 ml.) was placed in the main chamber of each cup immediately before gassing. Duplicate 1 ml. portions were analysed for total N. The total volume of fluid in each cup was 1·2 ml. Six manometers were used with each batch of Protozoa: two each to measure endogenous fermentation, glucose fermentation and test-substance fermentation. In the experiments designed to permit the analysis of galactose-fermentation products, the manometric procedure was modified as follows. The buffer solution used to suspend the organisms was the 'phosphate saline' of Heald & Oxford (1953), which contains no acetate or bicarbonate. The gas phase was O_2- and CO_2-free nitrogen. Warburg cups with two side arms were used; the first containing galactose (10μmoles in 0·2 ml. of buffer solution), and the second containing 10N-H_2SO_4 (0·2 ml.). The centre well contained 5N-NaOH (0·3 ml., carbonate-free). Acid was tipped in a control manometer at the beginning of the experiment, and in the others at the end, to stop the fermentation and to release CO_2.

Analytical methods. Total N in the protozoal suspensions was determined by a micro-Kjeldahl procedure. Reducing sugars were determined by Somogyi's (1945*a*) method, after clarifying with $ZnSO_4$ and baryta (Somogyi, 1945*b*). The aglucones produced during fermentation of the glucosides were detected by the colour reactions of Barnett, Ingram & Swain (1956). Paper chromatography of sugar solutions was as described by Howard (1957*b*). Lactic acid was determined by the method of Barker & Summerson (1941), and volatile acids by chromatography on buffered Celite columns (Bueding & Yale, 1951). Carbon dioxide in the alkali in the centre well of the Warburg cups was determined gravimetrically as $BaCO_3$. Storage polysaccharide in the cells was determined by reducing-sugar estimation after acid hydrolysis.

RESULTS

The results of a typical pair of manometric experiments, involving the fermentation of maltose by *D. ruminantium* and mixed isotrichs, are shown graphically in Fig. 1. The CO_2 constant, k_{CO_2}, was used to convert manometer readings into gas volumes, although the gas produced in the Warburg cups contained a small, and possibly variable, proportion of hydrogen. The reasons for using the CO_2 constant in this case have been discussed by Heald & Oxford (1953). In assessing the ease with which a substance was fermented, the rate of fermenta-

Table 1. *Rates of fermentation of carbohydrates by suspensions of* Dasytricha ruminantium *and* Isotricha *species*

See text for experimental conditions.

Substrate	*Dasytricha* Rate of fermentation (%)*	Sugar used (%)†	*Isotricha* Rate of fermentation (%)*	Sugar used (%)†
Galactose	70, 80, 67	96	0	1
Galactose‡	67	—	—	—
Maltose	33	52	0	0
Cellobiose	100	—	0	—
Aesculin	30, 50	§	4	§
Amygdalin	82, 93	—	13	—
Arbutin	60	§	28	§
Salicin	91	§	25	§
Sucrose	100	—	100	—
Raffinose	100	—	100	—
Xylose	0	1	0	—
Rice-starch grains	0	—	25	—
Holotrich starch: grains	0	—	3·2	—
Holotrich starch: defatted grains	0	—	3·3	—
Holotrich starch: precipitated	0	—	1	—

* Compared to glucose as 100%. † As % of glucose used in same period.
‡ Protozoa collected from rumen liquor by adding galactose instead of glucose.
§ Aglucone detected in the solution at end of experiment.

Table 2. *Products of fermentation of glucose and galactose by* Dasytricha ruminantium

See text for experimental conditions.

Product	Moles of product/100 moles of sugar used		Atoms of C (% of C in total breakdown products)	
	Glucose*	Galactose†	Glucose*	Galactose†
Glucose in cells	82‡	28·1	—	—
Carbon dioxide	23	92·1	21·5	23·0
Acetic acid	7·0	28·0	13·1	14·0
Butyric acid	4·8	12·9	17·7	12·9
Lactic acid	17	66·5	47·6	50·0

* Calculated from Table 1 of Gutierrez (1955). † Present results. ‡ Calculated by difference.

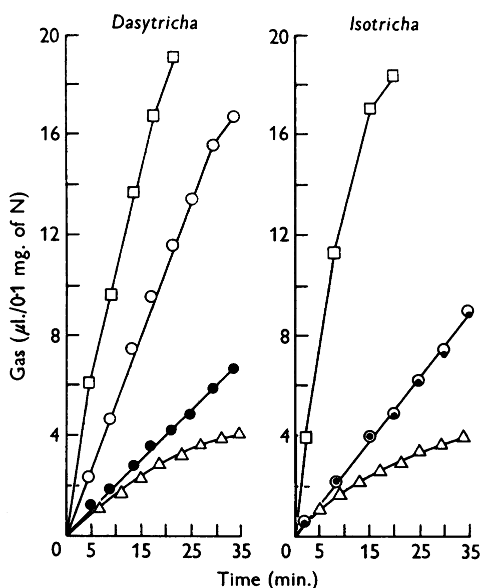

Fig. 1. Fermentation of sugars by the two genera of rumen holotrich Protozoa. For experimental conditions see text. ●, Endogenous fermentation; □, glucose; ○, maltose; △, glucosamine hydrochloride.

tion was compared with the endogenous fermentation and the fermentation of glucose, by portions of the same suspension. It was hoped in this way to allow for variations in metabolic activity between batches of Protozoa collected on different days. The rate of fermentation of a substance, corrected for the endogenous fermentation, could then be expressed as a percentage of the similarly corrected glucose-fermentation rate. A number of such values are shown in Table 1. Very high and very low values were reproducible between samples of Protozoa collected on different days, but where the rate of fermentation of a substance was intermediate the values sometimes differed markedly between samples.

In every case the rate of fermentation was linear during the period of observation, except for very rapidly fermented substances such as glucose, where exhaustion of substrate and developing acidity soon caused a falling-off in the rate of fermentation.

All the carbohydrates known hitherto as substrates for fermentation by rumen holotrichs are glucose, fructose and their derivatives. Galactose is the first exception to this generalization to be discovered, and it seemed of interest to investigate the products of metabolism of this sugar by *D. ruminantium*. An analysis of the contents of Warburg cups after such a fermentation is shown in Table 2. The reducing sugar present in acid hydrolysates of the cells before and after fermenting galactose was shown chromatographically to be almost wholly glucose; galactose was absent.

Mannose and glucosamine hydrochloride were not fermented by either type of holotrich, but were not metabolically inert, since a depression of endogenous fermentation was caused after a lag period. The effect of glucosamine hydrochloride is shown in Fig. 1; mannose produces a similar, but rather smaller, depression of endogenous fermentation. Microscopic examination at the end of the experiment showed the Protozoa exposed to mannose or glucosamine to be moribund, in contrast with the controls, which were normally active.

DISCUSSION

The conclusion, drawn from previous observations, that some biochemical differences exist between *Dasytricha* and *Isotricha* is confirmed by the present work. The fermentation of cellobiose by *Dasytricha*, but not by *Isotricha*, first observed by Gutierrez (1955) with organisms obtained from cattle in Pullman, Washington, U.S.A., has also been observed here with organisms from sheep. Whereas the *Dasytricha* studied in the present work fermented cellobiose approximately as rapidly as glucose, measurement of Gutierrez's (1955) fig. 1 suggests a fermentation rate for cellobiose of about 50% only, in the sense used in Table 1 above. Sugden & Oxford (1952) observed that adding

cellobiose to a mixed suspension of *D. ruminantium* and isotrichs prolonged the life of both genera. Since isotrichs alone cannot use cellobiose, it is evident that in this case they were profiting by the hydrolytic activity of *D. ruminantium*. The liberation into the medium of small amounts of cellobiase on the death and disintegration of *D. ruminantium* cells is the most likely explanation (see next paper, Howard, 1959). Eadie & Oxford (1955) described an abnormality of amylopectin storage that could be induced in rumen holotrichs while they were rapidly metabolizing a soluble sugar. When cellobiose was added to a mixed suspension, abnormality could be induced in *D. ruminantium* only, an observation explicable in the light of the present results.

Another example of the greater metabolic versatility of *Dasytricha* is the action of the two genera upon maltose. Here again, the isotrichs are unable to ferment this sugar, whereas *D. ruminantium* can do so, but not so quickly as glucose is fermented. When Heald *et al.* (1952) and Heald & Oxford (1953) tested mixed suspensions manometrically, no fermentation of maltose was observed. Apart from the low rate of maltose fermentation by mixed organisms which could be predicted from the data in Table 1, there is another factor which might have contributed to the diminution of activity. It has been noticed during the present experiments that when mixed suspensions are incubated for periods of about 48 hr., as in the experiments of Heald & Oxford (1953), the *D. ruminantium* tend to die off more rapidly than the isotrichs. A combination of these different factors might very well give rise to a suspension whose maltose-fermenting capacity is below what is measurable manometrically. The failure of Eadie & Oxford (1955) to induce abnormal amylopectin storage in either genus with maltose as substrate may be taken to indicate that fermentation approximately as rapid as that of glucose is required for the abnormality to be induced.

In fermenting the naturally occurring β-glucosides aesculin, amygdalin, arbutin and salicin, *Dasytricha* again has the advantage over *Isotricha*. In each case, the rate of fermentation by the former is several times that by the latter (Table 1). In contrast with its inability to ferment the β-glucoside cellobiose, *Isotricha* has an undoubted ability to make use of the glucose in the phenolic β-glucosides mentioned above, as is indicated not only by the gas production in the Warburg manometers but also by the detection of the aglucones in solution at the end of the experiment. The utilization of salicin for amylopectin synthesis by *D. ruminantium* but not *Isotricha* has already been noted by Gutierrez (1955); presumably the slow rate of fermentation by the latter does not allow of the accumulation of storage material.

When surveying infusoricidal compounds, Eadie, Mann & Oxford (1956) observed that not only was menthol (4 mM) very toxic to rumen holotrichs, but that both genera were killed by 6 mM-menthyl β-glucoside. The glucoside was toxic to the isotrichs even when no *D. ruminantium* were present, an observation which was inexplicable at the time, since it was concluded from the results of Gutierrez (1955) that no β-glucosidase was associated with *Isotricha*. The present work, showing that this genus does possess a definite small β-glucosidase activity, explains the apparent anomaly. In contrast with the toxicity of menthyl β-glucoside, no toxic effects of aesculetin, mandelonitrile, quinol or saligenin were noted in the present experiments, even though the concentration of aglucone that would be produced by complete hydrolysis of the glucosides is greater than 8 mM.

It was first observed by Sugden & Oxford (1952) and later by Gutierrez (1955) that *Isotricha* can ingest small starch grains, e.g. rice starch, but that the smaller *Dasytricha* cannot do so. That the rice-starch grains are truly fermented by the isotrichs but not by *D. ruminantium* is proved by the manometric experiments (Table 1). It is noteworthy that gas evolution in the manometers containing *Isotricha* commenced at the time of mixing; that is, the swallowing of, and biochemical attack on, the grains is very rapid. This agrees with the finding of Sugden & Oxford (1952) that the deposition of storage amylopectin granules was detectable within 5 min. of adding rice starch to a suspension of starved isotrichs. The same authors reported that granules of starch prepared from mixed rumen holotrichs were not swallowed or otherwise utilized by either *Isotricha* or *Dasytricha*. This was surprising, because the protozoal-starch grains are much smaller than rice-starch grains, and might be expected to be easily engulfed by *Isotricha*. This observation has been confirmed manometrically (Table 1). One possible explanation, that a lipid coating rendered the grains unpalatable, is excluded by the lack of activity of the Protozoa towards defatted grains. Eadie & Oxford (1955), contrary to the previous observation of Sugden & Oxford (1952), observed some ingestion of the protozoal-starch grains by *Isotricha*. It is clear that further investigation, both microscopical and biochemical, is required before these anomalies can be explained.

The products of galactose metabolism by *D. ruminantium* are identical with those formed from glucose by the same species (Table 2, columns 2 and 3). Particularly noteworthy is the conversion of a part of the galactose into glucose, as indicated by the increase in amylopectin content of the cells during fermentation. Perhaps because the galactose fermentation is slower than that of glucose, a much

smaller proportion is converted into amylopectin. Of the sugar converted into fermentation products proper, the proportions of the different products in the two cases are very similar (Table 2, columns 4 and 5). The most probable explanation of these results is that the galactose is first converted into glucose or a derivative. This hypothesis agrees with the results of studies of galactose metabolism by other organisms, where it has been shown (Leloir, 1955) that the following reactions are brought about by the action of the 'galactowaldenase' enzyme system: (1) uridine diphosphoglucose + galactose 1-phosphate ⇌ uridine diphosphogalactose + glucose 1-phosphate, (2) uridine diphosphogalactose ⇌ uridine diphosphoglucose.

The depression of fermentation caused by mannose and glucosamine, which was noted in the manometric experiments, agrees with the microscopic observations of Sugden & Oxford (1952), who found these two sugars, and also galactosamine, to be toxic to both genera of rumen holotrichs within 24 hr.

Further discussion of the present results will be found in the next paper (Howard, 1959).

SUMMARY

1. Suspensions of *Dasytricha ruminantium* and of mixed *Isotricha intestinalis* and *Isotricha prostoma* have been prepared from the rumen liquor of specially treated sheep, and used in manometric experiments.

2. The *D. ruminantium* suspensions, but not the isotrichs, were able to ferment galactose, maltose and cellobiose. Some naturally occurring β-glucosides were more readily fermented by *D. ruminantium* than by the isotrichs.

3. The products of galactose fermentation by *D. ruminantium* were the same as those formed from glucose by the same species.

4. Only the isotrichs could ferment rice-starch grains; neither genus could utilize starch grains from rumen holotrichs.

5. Previous observations on the rumen holotrichs, mostly made on mixtures of *Dasytricha* and *Isotricha*, are discussed in the light of these results.

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The Biochemistry of Rumen Protozoa

2. SOME CARBOHYDRASES IN CELL-FREE EXTRACTS OF *DASYTRICHA* AND *ISOTRICHA*

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The holotrich Protozoa of the rumen are known to possess a vigorous carbohydrate-fermenting system. In addition to glucose and fructose, a number of di-, tri- and poly-saccharides and glucosides can be fermented more or less readily (Heald & Oxford, 1953; Gutierrez, 1955; Howard, 1959). Cell-free extracts prepared by rupturing the protozoal cells have been found to contain invertase (Heald, Oxford & Sugden, 1952; Christie & Porteous, 1957) and amylase (Mould & Thomas, 1957). The last-named authors have also found in the extracts cellobiase, maltase and the enzymes responsible for the synthesis of starch (Mould & Thomas, 1958). They also achieved a partial separation of the two