

**Factors Affecting the Uptake and Metabolism
of Soluble Carbohydrates by the Rumen Ciliate
Dasytricha ruminantium Isolated from Ovine Rumen Contents
by Filtration**

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SUMMARY

A filtration technique is described whereby metabolically-active suspensions of *Dasytricha ruminantium* can be isolated from rumen contents with negligible contamination by bacteria or other protozoa. The effects of environmental factors and of the diurnal cycle of the rumen on the uptake and metabolism of soluble carbohydrates by these isolated cells were examined. The principal contribution of the protozoan metabolic end-products to the host ruminant is the supply of lactic, acetic and butyric acids during periods when soluble sugars are in excess.

INTRODUCTION

The holotrichous ciliated protozoa that occur in the rumen belong to the family Isotrichidae: two species of *Isotricha* and one species of *Dasytricha* are usually present. Their major fermentation products are lactic, acetic and butyric acids, carbon dioxide, hydrogen and storage polysaccharide (Howard, 1963). The protozoal polysaccharide has a highly-branched structure similar to that of amylopectin (Forsyth & Hirst, 1953).

The fermentation of soluble carbohydrates by *D. ruminantium* and *Isotricha* spp. has been examined *in vitro* using cultural studies (Sugden & Oxford, 1952), and washed preparations of either single genera (Guttierez, 1955; Howard, 1959*a*) or mixed holotrich species (Masson & Oxford, 1951; Heald & Oxford, 1953). Protozoa were separated from rumen liquor by sedimenting strained rumen contents (Heald, Oxford & Sugden, 1952) or by repeatedly washing the residue remaining after freshly-sampled rumen liquor had been filtered through surgical gauze (Oxford, 1951). *Dasytricha ruminantium* was subsequently recovered by differential sedimentation of the mixed holotrich preparation, or by making use of the tendency of the protozoa to adhere to the inner walls of the glassware used in the initial sedimentation (Guttierez, 1955). Partial defaunation of the host animal permits the introduction of specific protozoan species into the rumen simplifying the recovery process (Eadie & Oxford, 1957; Howard, 1957). As each of these methods has disadvantages, a filtration method has been developed for isolating metabolically-active preparations of *D. ruminantium*.

The rate of glucose fermentation (Guttierez, 1955) and the range of soluble carbohydrates metabolized by *D. ruminantium* (Howard, 1959*a*) have been determined manometrically. Although the enzymic pathways between carbohydrate substrate and end product have

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not been characterized, the carbohydrase activity of cell-free *Dasytricha* preparations has been examined (Howard, 1959*b*; Abou Akkada & Howard, 1961; Bailey & Howard, 1963). However, the extent to which environmental conditions affect carbohydrate metabolism has not been assessed. Therefore, aspects of the carbohydrate metabolism of *Dasytricha* preparations recovered by filtration were examined and compared with the data already available. This work was briefly reported earlier (Williams & Harfoot, 1975).

METHODS

Preparation of protozoal suspensions. Rumen liquor was obtained from two three-year-old Suffolk cross Blackface sheep fitted with permanent rumen fistulae. The sheep were given 0.6 kg cubed molassed sugar-beet pulp at 07.00 h daily and 0.3 kg chopped hay at 16.00 h daily. Water was given *ad lib.* On this diet a stable rumen protozoal population was established, consisting of (organisms ml⁻¹): *D. ruminantium*, 3000 to 5000; mixed *I. prostoma* and *I. intestinalis*, 700 to 1000; and entodiniomorphs, 25000 to 30000.

The rumen liquor sample was strained through a double layer of surgical gauze, diluted twofold with the inorganic salts solution and transferred to a separating funnel where it was allowed to stand for 30 to 60 min at 39 °C. The particulate material retained by filtration was washed with the salts solution and the washings were combined with the diluted liquor. The inorganic salts solution contained (g/l distilled water): NaCl, 5.0; KH₂PO₄, 0.3; K₂HPO₄, 1.0; MgSO₄·7H₂O, 0.1; CaCl₂·2H₂O, 0.1; Na₂S·9H₂O, 1.0; final pH 7.0. All solutions, media and glass vessels used in the experiments were gassed with oxygen-free N₂ and warmed to 39 °C before use.

During sedimentation, the holotrichs settled to the bottom of the funnel, and they were collected in fresh buffer. The addition of glucose to aid sedimentation (Oxford, 1951) was unnecessary, and the endogenous fermentation of the liquor was sufficient to cause flocculant debris to rise to the surface as a scum layer. Similar separation was obtained, without adding glucose, using liquor samples obtained from sheep given hay or hay and concentrate diets, though the collection time was increased to 90 min for samples withdrawn immediately before the morning feed.

The protozoal pellet thus obtained, which consisted of mixed holotrichs, entodiniomorphs (*Dasytricha*:*Isotricha*:entodiniomorphs, approx. 9:1:10) and bacteria, was washed on a sintered-glass filter (pore size 40 to 90 μm; 140 mm diam.; Sintaglass, Gallenkamp, London). *Isotricha* spp. and large entodiniomorphs (*Eudiplodinium* and *Polyplastron*) were retained by the filter, and *D. ruminantium* was recovered from the filtrate by washing and differential sedimentation. Before washing and sedimentation, the ratio of *Dasytricha* to small entodiniomorphs in the filtrate was approximately 20:1, but differential microscopic counts on washed suspensions indicated that the entodiniomorph contamination was less than 0.1%, and *Isotricha* and large entodiniomorphid protozoa were absent. The *D. ruminantium* cells recovered were morphologically normal and fully active. Viable preparations were maintained in a supplemented buffer solution for long periods (24 to 48 h).

Isotricha spp. were separated from the accompanying *Polyplastron* remaining on the filter by repeated washing, using a separating funnel and allowing the *Isotricha* to adhere to the inner walls of the glass vessel in a manner analogous to that described for *D. ruminantium* by Gutierrez (1955).

Experiments involving uptake of radioactive substrates. The rate of glucose incorporation was determined in sealed Erlenmeyer flasks under a N₂ atmosphere in a supplemented buffer

solution (SBS) containing (mg ml⁻¹): K₂HPO₄, 8.6; KH₂PO₄, 6.8; NaCl, 5.0; MgSO₄·7H₂O, 0.1; CaCl₂·2H₂O, 0.1; dithiothreitol, 0.25; and glucose, 5.0, containing D-[U-¹⁴C]glucose (0.1 μCi ml⁻¹); pH 7.5. The buffer solution was gassed with N₂ and equilibrated to 39 °C before inoculation. Samples of the incubation mixture were withdrawn at timed intervals through an airtight seal (Suba-Seal, Gallenkamp, London) using a hypodermic syringe, and the cells were killed by immediate transfer to an equal volume of formalin (4 %, v/v) at 4 °C. The protozoa were recovered by centrifuging (500g; 5 min), washed twice with, and re-suspended in, distilled water. Uptake studies using D-[U-¹⁴C]fructose, [U-¹⁴C]sucrose, D-[U-¹⁴C]mannose and D-[1-¹⁴C]galactose were performed similarly using 5 mg ml⁻¹ (0.1 μCi ml⁻¹) of the labelled substrate.

Determination of acidic products of fermentation. Lactate and volatile fatty acid (VFA) production were also determined in sealed Erlenmeyer flasks under a N₂ atmosphere at 39 °C in the SBS buffer, pH 7.5. Unlabelled carbohydrate substrates (5.0 mg ml⁻¹) were used, and the samples, removed at timed intervals as described above, were immediately centrifuged (500g; 5 min) at 4 °C. The protozoa were killed in formalin and prepared for analysis as described above, and the cell-free supernatant was assayed for VFA, lactate and sugar. Endogenous fermentation rates were determined in an identical manner in the absence of an exogenous carbon substrate.

Manometric methods. The rate of gas production was determined under a N₂ atmosphere at 39 °C using Warburg manometry, and a Gilson differential respirometer (Gilson, Villiers le Bel, France). The main compartment of the reaction vessels contained 2.5 ml SBS buffer pH 7.5 and 0.5 ml inoculum (0.4 to 2.0 mg protein), and the glucose substrate (20 mg in 1.0 ml SBS buffer) was added from the side-arm unit. The Warburg manometer readings were converted into gas volumes using the reaction vessel K_{CO₂} constant (Heald & Oxford, 1953).

Analytical methods. Acetate and butyrate were estimated directly in the acidified incubation mixture supernatants using a Pye Unicam 104 gas chromatograph fitted with a flame ionization detector and 1.6 m stainless-steel columns packed with 5 % Carbowax 20M TPA supported on acid-washed Chromosorb G previously treated with dimethylchlorosilane (Perkin-Elmer, Beaconsfield, Buckinghamshire). The column temperature was maintained isothermally at 140 °C and the detector temperature at 200 °C. The carrier gas was oxygen-free N₂ maintained at a flow rate of 60 ml min⁻¹. The concentrations of acetic and butyric acids were determined by adding a known amount of crotonic acid to each sample (Cottyn & Boucque, 1968).

Lactic acid and glucose were determined enzymically using lactic acid dehydrogenase (Boehringer Corp., London) and glucose oxidase (Sigma) respectively.

Total protein and total carbohydrate were estimated in cell lysates prepared by heating the washed suspensions in equal volumes of 1 M-NaOH at 100 °C for 5 min. Protein was estimated colorimetrically by the Folin-Ciocalteu method (Lowry *et al.*, 1951) and carbohydrate by the phenol-sulphuric acid method (Dubois *et al.*, 1956).

All radioactive determinations were made using a Packard Tri-Carb model 2425 liquid scintillation spectrometer. Samples of the lysates were counted as emulsions in Unisolve I liquid scintillator (Koch-Light).

Chemicals. All chemicals were of Analar grade. All radioactive substrates were purchased from The Radiochemical Centre, Amersham, Buckinghamshire.

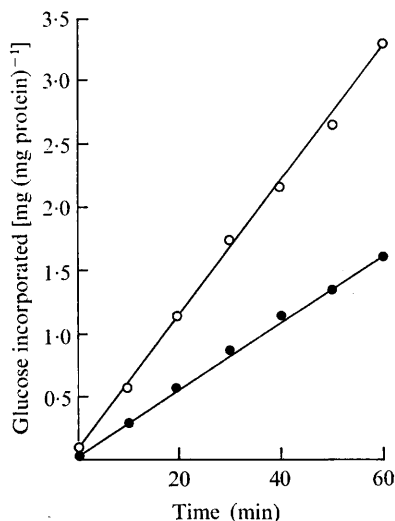


Fig. 1. Glucose incorporation by washed suspensions of *D. ruminantium* (●) and mixed *I. intestinalis* and *I. prostoma* (○), determined in SBS with [¹⁴C]glucose.

Table 1. *Effect of glucose concentration on glucose uptake and metabolite formation by D. ruminantium*

The rates measured (glucose, 5 mg ml⁻¹; protein, 0.2 mg ml⁻¹) were [(mg protein)⁻¹ h⁻¹]: glucose uptake, 1.54 mg; lactate formation, 7.66 μmol; butyrate formation, 1.30 μmol; gas formation, 118 μl. The results are expressed as percentages of these values.

Glucose concn (mg ml ⁻¹)	Glucose incorporation rate	Metabolite production rate		
		Lactic acid	Butyric acid	Gas
0.1	15.6	5.10	74.6	50.4* 30.0†
0.25	25.3	34.6	96.9	90.2* 28.6†
0.5	44.8	57.7	103.1	105.5* 33.8†
1.0	72.1	61.9	106.9	98.2
2.5	101	100.6	106.9	95.4
5.0	100	100	100	100
10	103	104	100	102.7
Endogenous	—	6.8	42.3	29.4

* Initial rate.

† Rate after 60 min incubation.

RESULTS

Effect of incubation conditions on the rate of incorporation of [¹⁴C]glucose by suspensions of holotrich protozoa

The rate of incorporation of ¹⁴C-labelled glucose into the cellular components of *D. ruminantium* at 39 °C and at pH 7.5 was 1.4 to 1.6 mg glucose (mg protein)⁻¹ h⁻¹ under optimal experimental conditions (Fig. 1). The rate was independent of the glucose concentration above 2.5 mg ml⁻¹, when using approximately 0.1 to 0.2 mg protein ml⁻¹ (Table 1). At more than 10 mg ml⁻¹, glucose damaged the cells osmotically. The amount of [¹⁴C]glucose incorporated was directly proportional to the cell protein in the incubation system over the ranges examined (0.01 to 0.1 and 0.1 to 1.0 mg protein ml⁻¹). However, when the amount of protein present in the incubation mixture exceeded 2.0 mg ml⁻¹, the low environmental

pH which developed, owing to the accumulation of acidic end-products, often resulted in death. Similarly, the presence of oxygen led to lysis. The rate of glucose incorporation was not affected by the presence of streptomycin sulphate at bactericidal concentrations of 1.0 mM.

Under similar conditions, *Isotricha* spp. incorporated 3.1 mg glucose (mg protein)⁻¹ h⁻¹ (Fig. 1).

Distribution of ¹⁴C from [U-¹⁴C]glucose incorporated into the cell

To determine the distribution of ¹⁴C within the protozoa, a sample of the washed cell suspension removed from the incubation flask after 60 min was fractionated with trichloroacetic acid (TCA) as described by Coleman (1969). A similar sample was refluxed with CHCl₃/CH₃OH (2:1, v/v) to extract the lipids. The CHCl₃/CH₃OH extracts were evaporated to dryness and dissolved in 0.4% (w/v) Scintimix 4 scintillator (Koch-Light) in toluene. The TCA-treated fractions were counted as aqueous emulsions in Unisolve. The distribution of the radioactivity derived from [¹⁴C]glucose was as follows: lipid material, < 0.5%; protozoal metabolite pool (cold TCA-soluble), 2 to 3%; protein (hot TCA-insoluble), 3 to 5%; and polysaccharide (hot TCA-soluble), 90 to 95%. The distribution was not affected by the preparative procedure used: the following values were obtained in cells from the same incubation which were, respectively, either fixed and washed as described, or washed in salts medium: lipid, < 0.5% and < 0.5%; metabolite pool, 2.1% and 1.9%; protein, 3.1% and 4.0%; polysaccharide, 94.3% and 93.6%. However, the radioactive products derived from [1-¹⁴C]acetate were fairly evenly distributed between the metabolite pool (25 to 35%), lipid (20 to 30%), and protein (25 to 35%), with a smaller proportion appearing in the polysaccharide fraction (15 to 20%). These observations are consistent with the findings of Coleman (1969) using rumen entodiniomorph protozoa and Harmeyer & Hekimoglu (1968) using *Dasytricha*.

Effect of pH and temperature on the rate of glucose incorporation

The optimum pH range for the incorporation of [¹⁴C]glucose by *Dasytricha* was pH 7 to 8, where the rate of incorporation was approximately 1.5 mg glucose (mg protein)⁻¹ h⁻¹. At the extremes of pH tolerated by the organism (pH 5 and pH 9), this rate of incorporation was reduced by 55% and 65% respectively (Fig. 2). These findings confirm the observation by Quinn, Burroughs & Christiansen (1962) that rumen protozoa will not survive exposure to pH values outside the range pH 5 to 9, and have maximum activity at pH 7 to 8. The optimum temperature for the incorporation of glucose by *Dasytricha* was about 40 °C. The organism lysed at temperatures in excess of 50 °C; below 40 °C it survived, but incorporation was much slower (Fig. 3). The accepted temperature of the rumen environment is 39 to 40.5 °C (Hungate, 1966).

*Fermentation of glucose by *Dasytricha**

As well as being converted into storage polysaccharide, glucose was fermented to acetic, butyric and lactic acids and gas (Fig. 4). Small amounts of propionic acid were occasionally detected. Typical rates of formation are given in Table 2. The rates of gas production were similar to those observed by Howard (1959*a*) and values for total acid production were in close agreement with those given by Gutierrez (1955), confirming that the *Dasytricha* and *Isotricha* suspensions prepared using the filtration method were metabolically comparable with the preparations used in other studies *in vitro*. The rate of acid production was directly proportional to the amount of cell protein present. The average proportions of

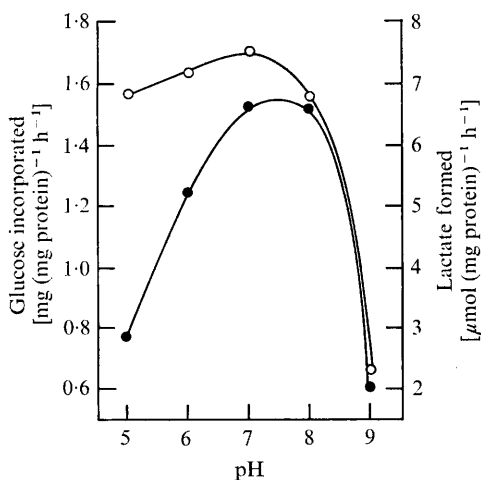


Fig. 2

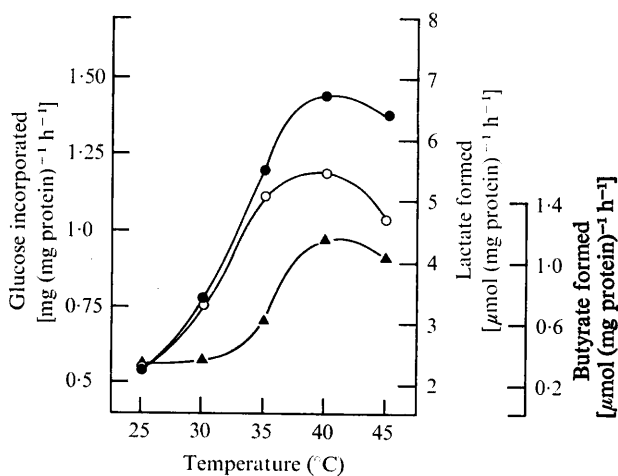


Fig. 3

Fig. 2. Effect of incubation pH on the rate of glucose incorporation (●) and lactate formation (○) by *D. ruminantium*. The initial pH values were stabilized by the following buffer systems (all prepared in SBS): pH 5 to 7, McIlvaine citrate-phosphate; pH 8 and 9, 0.1 M-Tris/HCl. When pH 7 was stabilized by 0.1 M-KH₂PO₄/K₂HPO₄, the results were identical.

Fig. 3. Effect of incubation temperature on the rate of glucose incorporation (●), and the formation of lactic acid (○) and butyric acid (▲) by *D. ruminantium*.

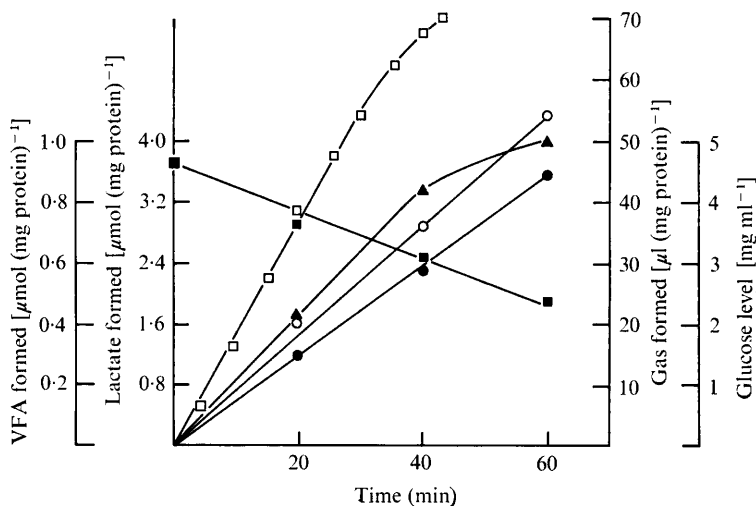


Fig. 4. Production of fermentation products from glucose (■) by *D. ruminantium*: acetic acid (●), butyric acid (○), lactic acid (▲) and gas (□).

metabolic end-products from glucose present at 5 mg ml⁻¹ in 10 incubations were estimated to be: acetic acid, 2.3 % (1.2 %); butyric acid, 3.7 % (2.5 %); lactic acid, 19.8 % (18.8 %); carbon dioxide, 6.0 % (4.5 %), assuming CO₂ and H₂ are produced in 1:1 proportion (Heald & Oxford, 1953; Gutierrez, 1955); and storage polysaccharide, 68.2 % (73 %). The figures in parentheses are the proportions formed after corrections for endogenous metabolism have been made. With glucose at less than 2.5 mg ml⁻¹, the metabolites were formed more

Table 2. Fermentation data, both endogenous and when incubated in SBS containing glucose (5.0 mg ml⁻¹), for *D. ruminantium* recovered from the rumen at various times throughout the diurnal cycle

The figures quoted are the mean of at least two separate incubations. Carbon recoveries were within the range 95 to 105 %. Gas production rates were determined using Warburg manometry.

Time after feeding sugar-beet pulp (h) ...		1	2	3	4	5	7	9	15	21	24
Glucose incorporated [mg (mg protein) ⁻¹ h ⁻¹]		1.52	1.48	1.38	1.47	1.56	1.38	1.56	ND	ND	1.49
Lactate formed [μmol (mg protein) ⁻¹ h ⁻¹]	Endogenous	0.42	0.37	0.49	0.42	0.45	0.50	0.47	ND	ND	0.10
	Glucose present	4.31	5.78	4.80	5.39	5.07	3.98	4.00			3.80
Acetate formed [μmol (mg protein) ⁻¹ h ⁻¹]	Endogenous	0.49	0.51	0.37	0.50	0.42	0.64	0.53	ND	ND	0.20
	Glucose present	1.17	0.92	0.87	0.83	0.61	0.82	0.66			0.94
Butyrate formed [μmol (mg protein) ⁻¹ h ⁻¹]	Endogenous	0.38	0.60	0.22	0.33	0.16	0.43	0.36	ND	ND	0.07
	Glucose present	0.83	0.95	0.75	1.01	0.68	1.11	0.96			0.72
Gas formed [μl (mg protein) ⁻¹ h ⁻¹]	Endogenous	28.1	27.4	46.0	26.6	26.0	30.5	45.9	ND	ND	24.0
	Glucose present	122.8	130.7	140	122.2	121.3	136.1	142.8			97.5
Cell carbohydrate: cell protein (mg mg ⁻¹)	*	1.08	1.55	1.63	1.43	1.39	1.28	1.22	0.83	0.47	0.42
	†	2.37	2.98	2.67	2.55	2.79	2.32	2.42	ND	ND	1.67
Total soluble rumen carbohydrate (mg ml ⁻¹)		0.54	0.44	0.37	0.35	0.33	0.27	0.27	0.22	0.20	0.22

ND, Not determined.

* Immediately on recovery from rumen.

† After 60 min incubation in the presence of glucose (5 mg ml⁻¹).

slowly (Table 1). In incubations in which the levels of glucose (0.5 mg ml⁻¹) and *Dasytricha* protein (0.02 mg ml⁻¹) resembled those *in vivo* 1 h after feeding, the measured rates of glucose uptake and metabolite production were identical to those determined in the standard incubation system, i.e. glucose 5.0 mg ml⁻¹, *Dasytricha* protein 0.2 mg ml⁻¹.

The maximum rate of lactic acid (Fig. 2) and VFA formation occurred at pH 7. The effect of pH on the rate of formation of lactic acid and the volatile fatty acids, acetate and butyrate, was similar.

The optimum temperature for the production of both lactic and butyric acids was about 40 °C; and the rates of production of acetic and butyric acids showed similar variation with temperature.

Endogenous fermentation by Dasytricha

The *Dasytricha* obtained from the rumen contained varying amounts of storage polysaccharide. In order to determine the rate of fermentation of this polysaccharide, suspensions of the organism were incubated in the absence of added substrate. Typical mean endogenous rates of formation obtained using cells recovered after the morning feed were [μmol (mg protein)⁻¹ h⁻¹]: lactic acid, 0.45 (range 0.3 to 0.6); acetic acid, 0.50 (0.3 to 0.7); butyric acid, 0.35 (0.2 to 0.7). The endogenous rate of gas production was approximately 0.9 to 2.3 μmol (mg protein)⁻¹ h⁻¹.

Dasytricha cells taken from the rumen before the morning feed were incubated with [¹⁴C]glucose for 30 min at 39 °C, recovered by centrifuging and washed free of labelled substrate. They were then suspended in SBS buffer solution at 39 °C and the radioactivity

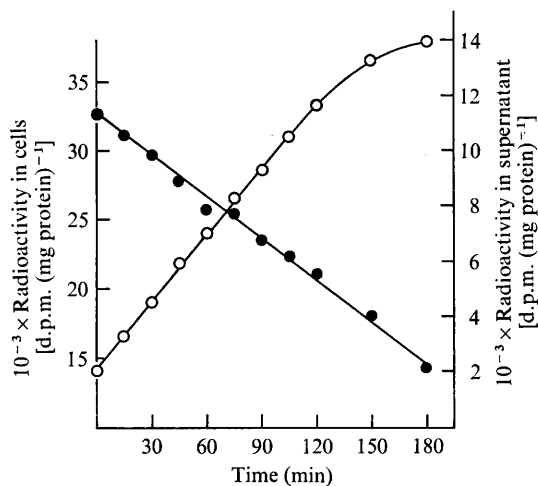


Fig. 5

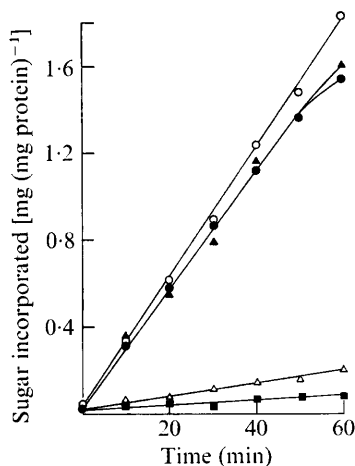


Fig. 6

Fig. 5. Fermentation of ^{14}C -labelled storage polysaccharide (●) and production of metabolites (○) endogenously by *D. ruminantium*.

Fig. 6. Uptake of ^{14}C -labelled carbohydrates by *D. ruminantium*: fructose (○), sucrose (▲), glucose (●), galactose (△) and mannose (■).

present in both the cells and the suspending solution was measured at intervals over a 3 h period (Fig. 5). The linear decrease in radioactivity associated with the cells [$6000 \text{ d.p.m. (mg protein)}^{-1} \text{ h}^{-1}$] indicated continuous utilization of storage polysaccharide, while the concomitant increase in radioactivity in the supernatant [$4900 \text{ d.p.m. (mg protein)}^{-1} \text{ h}^{-1}$] indicated the production of labelled metabolites following this utilization. The rates of lactic, acetic and butyric acid production were 0.08 , 0.25 and $0.18 \mu\text{mol (mg protein)}^{-1} \text{ h}^{-1}$ respectively. The 18.3% difference between the rate of loss of radioactivity from the cells and the rate of increase in the supernatant can presumably be related to carbon dioxide formation, in that the proportions of metabolic end-products formed during the endogenous fermentation of storage polysaccharide were estimated to be: acetic acid, 24%; butyric acid, 23%; lactic acid, 34%; and CO_2 , 19% (assuming CO_2 and H_2 are produced in 1:1 proportion).

Effect of the diurnal cycle of the rumen on the metabolic activity of Dasytricha

Cells were recovered from rumen samples taken at intervals up to 24 h after the sheep had been fed sugar-beet pulp. The rates of metabolism of the cells in the absence and presence of glucose were determined, as were the carbohydrate:protein ratios of the organisms (Table 2).

The endogenous metabolic activity was reduced in cells depleted of storage polysaccharide; although the subsequent rate of uptake of glucose, and other metabolic features, were similar throughout the cycle. The carbohydrate:protein ratio of the cells from the rumen did not approach that observed following incubation of the cells with glucose. The increase in the carbohydrate:protein ratio measured directly agreed closely with the value extrapolated from the data obtained in incubations with [^{14}C]glucose.

Table 3. Acid and gas production by *D. ruminantium* from a range of soluble carbohydrates (5.0 mg ml⁻¹) incubated in SBS

The protozoa were isolated from the sheep 2 h after the morning (sugar-beet) feed. The rates were measured as μ l gas produced (mg protein)⁻¹ h⁻¹ or μ mol acid produced (mg protein)⁻¹ h⁻¹, but are presented here as a percentage of the value obtained with glucose. Gas formation rates were measured using a Gilson Differential Respirometer.

Carbohydrate	Relative rates of formation			
	Gas	Acetic acid	Butyric acid	Lactic acid
D-Glucose	100	100	100	100
L-Glucose	37.1	25.9	42.6	18.2
Galactose	80.6	59.1	54.5	27.6
Fructose	114.6	97.1	91.5	101.6
Mannose	76.3* 26.1†	12.8	40.8	16.2
Glucosamine	41.2* 16.4†	13.7	39.6	12.3
Sucrose	109.6	89.4	105.7	100.0
Cellobiose	103.2	69.2	54.3	57.1
Maltose	89.1	49.1	62.1	38.1
Lactose	59.4	63.3	52.8	20.4
Maltotriose	77.5	57.6	50.9	34.6
Raffinose	104.4	103.5	97.1	84.6
Pectin	117.4	62.2	84.5	90.0
Carboxymethylcellulose	49.6* 34.5†	25.2	47.6	21.1
Inulin	109.6	89.8	80.4	106.3
Endogenous	37.1	25.6	45.2	18.2

* Initial rate.

† Rate after 60 min incubation.

Uptake and metabolism of sugars other than glucose by D. ruminantium

The average rates of uptake of ¹⁴C-labelled glucose, fructose, sucrose, galactose and mannose were, respectively, approximately 9.0, 9.0, 4.7, 1.2 and 0.45 μ mol sugar (mg protein)⁻¹ h⁻¹ (Fig. 6). The incorporation of fructose and sucrose [1.5 to 1.8 mg (mg protein)⁻¹ h⁻¹] was often up to 10 % faster than the measured rate of glucose incorporation. There was extensive lysis of cells incubated with mannose. Lactic acid, VFA and gas production from these sugars and a range of soluble saccharides is shown in Table 3. The compounds utilized were derived from glucose, galactose and fructose. The protozoa, although readily utilizing D-glucose, did not metabolize L-glucose. Similarly β -methyl-D-glucoside increased metabolic activity whereas neither α -methyl-D-glucoside nor 2,3,4,6-tetramethyl-glucose were apparently utilized. Other derivatives which did not increase the endogenous metabolism were the hexoses α -glucose 1-phosphate, α -glucose 6-phosphate, glucosamine hydrochloride, galacturonic acid and rhamnose, the polymers polygalacturonic acid, galactan and methyl cellulose, the pentoses ribose, xylose and arabinose, and the hemicellulosic polysaccharides xylan and araban.

Carboxymethylcellulose, glucosamine and mannose initially increased the rate of gas production, but this rate returned to the endogenous value with carboxymethylcellulose and fell below the endogenous rate with the monosaccharides. The fermentation of pectin (0.5 to 2.5 mg ml⁻¹) led to initial rates of gas formation which were similar to, or higher than, the comparable glucose fermentation rates, but which were not maintained and rapidly fell to the endogenous values. Metabolite production rates were maintained with pectin at 5 mg ml⁻¹.

DISCUSSION

The filtration technique has been used with consistent success to prepare metabolically-active suspensions of *D. ruminantium*. The method has distinct advantages over other procedures used for the isolation of rumen protozoa. Suspensions of *Dasytricha* with negligible contamination by bacteria or other protozoan species can be prepared in high yields, whereas differential sedimentation often results in incomplete separation of the holotrich species. Methods relying on the adhesion of *Dasytricha* to glass surfaces (Gutierrez, 1955) are unreliable and produce poor yields. Using the filtration technique to isolate *Dasytricha*, partial defaunation of the host animal is unnecessary (Eadie & Oxford, 1957), although in combination the two techniques should permit the establishment and recovery of pre-determined rumen protozoan species.

The technique is especially applicable to the preparation of protozoan suspensions for metabolic studies, in view of the sensitivity of these organisms to excessive manipulation. The method is rapid, in that the separation process can be completed within 1 h of withdrawing the rumen contents or, where small volumes of rumen contents are involved (< 200 ml), the intermediate sedimentation procedures can be eliminated and the liquor filtered directly. Also, as the cells are recovered without recourse to preliminary exposure to an exogenous fermentable carbon source (Heald *et al.*, 1952), metabolic studies may be conducted on cells having a metabolic status similar to that *in vivo*. As no additional polysaccharide material is formed during the isolation procedure, metabolic studies may be carried out without preliminary starvation (Heald & Oxford, 1953). It is also possible that the procedure may be applicable to the separation of other species of rumen protozoa using one or more filters of different porosities.

The rate of glucose uptake was not affected by the presence of reserve polysaccharide within the cells, the concentration of glucose in the incubation medium once in excess, or the diurnal cycle of the rumen. With excess substrate, the protozoa accumulated sufficient storage polysaccharide to rupture the cell, indicating that *Dasytricha* may lack a mechanism for controlling the entry of substrate into the cell. The rate of incorporation of mannose was low and the extensive cell lysis in its presence was not related to any excessive formation of reserve polymer (Sugden & Oxford, 1952).

The protozoon utilizes as carbon sources D-glucose, D-fructose and certain of the di-, tri-, and polysaccharides formed from them. Using manometric techniques Heald & Oxford (1953) did not detect fermentation of galactose, but Howard (1959*a*) reported that his preparation of *Dasytricha* utilized galactose at 70 % of the rate of glucose utilization. We found that the observed rate of [^{14}C]galactose incorporation was only 10 to 15 % of the rate of glucose utilization, whereas the rates of production of acetic, butyric and lactic acids and total gas from galactose were, respectively, approximately 59 %, 33 %, 18 % and 80 % of the rates from glucose, indicating that more than one criterion should be considered in assessing the rate of utilization of a substrate. The utilization of maltose, lactose or maltotriose also resulted in the formation of low levels of lactic acid, although gas production was only slightly less than when glucose was fermented.

The restricted carbohydrase activity of *D. ruminantium* limits the range of disaccharides that it can utilize. Invertase (Christie & Porteous, 1957; Carnie & Porteous, 1959), cellobiase, maltase and β -glucosidase (Howard, 1959*b*) activities have been detected in cell-free extracts of *Dasytricha*. Although *Dasytricha* possesses both pectin esterase and polygalacturonase activity (Wright, 1960; Abou Akkada & Howard, 1961), polygalacturonic and galacturonic acids were not effectively utilized during incubation.

Assuming there to be about 3000 *Dasytricha* ml⁻¹ in a sheep rumen of 5 l, and using the value of 6.6 ng estimated by Guttierrez (1955) for the amount of protein in a single *Dasytricha* cell, the ovine rumen is estimated to contain approximately 100 mg *Dasytricha* protein. The soluble sugar levels throughout the diurnal cycle are maintained at a level sufficient to raise fermentation above the endogenous rate only for some 2 to 3 h after feeding sugar-beet pulp. As the mean endogenous rates of production of lactic, acetic and butyric acids are ($\mu\text{mol h}^{-1}$) 45, 50 and 35 respectively, and 400 to 500, 90 to 120 and 80 to 100 respectively for the period when soluble sugars are in excess, these acids represent the principal contribution of the organisms to the ruminant. The carbohydrate and protein made available to the host depend on the rate of wash-out from the rumen. Weller & Pilgrim (1974) suggest that there may be considerable sequestration of protozoa in the rumen. This, with the results obtained above, lends support to the suggestion of Heald (1951) that *Dasytricha* polysaccharide makes little contribution to the total carbohydrate available to the host.

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