The Metabolism of Cellulose, Glucose and Starch by the Rumen Ciliate Protozoon *Eudiplodinium maggii*

By GEOFFREY S. COLEMAN

Biochemistry Department, Agricultural Research Council,
Institute of Animal Physiology, Babraham, Cambridge CB2 4AT

(Received 8 February 1978)

Washed suspensions of the rumen ciliate protozoon *Eudiplodinium maggii* incubated anaerobically in the presence of ampicillin utilized cellulose and to a lesser extent starch, but not soluble sugars. The protozoon incorporated ^{14}C from ^{14}C -labelled cellulose, glucose and starch; it synthesized protein from these compounds but only at a rate that would allow for the protozoon to divide every 8300, 1200 and 580 h, respectively, if this were the sole source of protein. These substrates were also metabolized with the production of acetic, propionic and butyric acids. Evidence is presented that 70 % of the cellulase inside the protozoa is soluble and not of bacterial origin. It had an activity of 11 to 28 pg cellulose digested h^{-1} protozoon $^{-1}$ [0.55 to 3.3 μg cellulose digested h^{-1} (mg protein) $^{-1}$].

INTRODUCTION

Hungate (1942) first grew *Eudiplodinium maggii* *in vitro* in the presence of bacteria and showed, by microscopical examination of starved protozoa, that they converted cellulose into 'paraglycogen' and that protozoal extracts produced sugar from cellulose. Recently, *E. maggii* has been shown to grow better on ground, dried grass than on wholemeal flour and to contain enzymes that liberate soluble ^{14}C -labelled compounds from an insoluble preparation of [^{14}C]cellulose (Coleman *et al.*, 1976). The present paper describes the metabolism of [^{14}C]cellulose, [^{14}C]starch grains and [^{14}C]glucose by washed suspensions and extracts of *E. maggii* grown *in vitro* or as the only ciliate protozoon in the rumen of a sheep.

METHODS

Organism. *Eudiplodinium maggii* was grown *in vitro* on ground, dried grass as the sole source of carbon, apart from contaminating bacteria, as described by Coleman *et al.* (1976). The work was carried out with four separate isolates (co2, 4L, c and w) all of which behaved similarly, although they varied in size from 102 $\times$ 62 μm (isolate c) to 150 $\times$ 75 μm (isolate co2). Washed suspensions were prepared as described by Coleman (1969) except that mineral salt solution D [containing (g l^{-1}): K_2HPO_4 , 6.3; KH_2PO_4 , 5.0; NaCl , 0.65; CaCl_2 (dried), 0.045; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.09] was used throughout. All suspensions inevitably contained some grass since this protozoon sedimented at almost the same rate as the plant material on which it was grown.

As the protozoal population density in culture was 100 to 200 ml^{-1} , it was difficult to obtain sufficiently large numbers of protozoa for some metabolic experiments. For these, the protozoa were harvested from the rumen contents of a Clun Forest sheep (fed on 800 g hay and 100 g oats daily) that had been treated initially with the dioctyl ester of sodium sulphosuccinate (Abou Akkada *et al.*, 1968) to kill all the ciliate protozoa and then refaunated with *E. maggii* from a laboratory culture. To harvest the protozoa, rumen contents were strained through one layer of muslin and then allowed to stand for 30 min in a separating funnel at 39 °C. The protozoa which sedimented to the bottom of the funnel were run off and washed at least six times by gentle centrifugation (on a swing-out-head centrifuge for 20 s from rest, maximum speed equivalent to 200 g) in salt solution D at room temperature. As much as possible of the green layer that

sedimented above the protozoa was removed. Rumen- or *in vitro*-grown protozoa were finally resuspended in salt solution D containing 0.1 % (w/v) L-cysteine hydrochloride (neutralized with NaOH) and bubbled for 2 min with N_2/CO_2 (95:5, v/v) before use.

Incubation conditions. The medium contained: 0 to 0.2 ml ^{14}C -labelled cellulose, starch grains or glucose; 0 to 0.1 ml 0.3 % (w/v) chloramphenicol or 1.0 % (w/v) ampicillin; 1.0 ml suspension (500 to 100000 ml $^{-1}$) of intact or, where indicated, sonicated protozoa; and 0 to 0.45 ml salt solution D containing 0.1 % cysteine hydrochloride, to give a final volume of 1.5 ml. Thick-walled centrifuge tubes (100 × 13 mm) were used; after inoculation N_2/CO_2 (95:5, v/v) was bubbled through the medium for 15 s at room temperature and the tube was then sealed with a rubber bung and incubated at 39 °C. For experiments on the survival of protozoa, unlabelled substrates were used and extra salt solution D containing 0.1 % cysteine hydrochloride was added to make the final volume 3.0 ml.

After incubation, intact protozoa were gently centrifuged (see above) and washed (× 3) in salt solution D at room temperature. The washed protozoa were then either plated out for the determination of radioactivity (Coleman, 1969) or suspended in 2 ml H_2O and disrupted (see below). Any bacteria present and most of the cellulose or starch grains that had not been engulfed were sedimented from the first supernatant fluid obtained after removal of the protozoa and washed once (2000 g, 20 min, 4 °C, on an angle-head centrifuge). Samples from the supernatant fluid after removal of the bacteria were placed on planchets with 0.1 ml 1 M-HCl or 0.1 ml 0.1 M-NaOH for estimation of ^{14}C which was free in the medium. The radioactivity determined with HCl measured the non-volatile ^{14}C and the difference between the determinations in the presence of HCl and NaOH measured the ^{14}C in volatile fatty acids.

Disruption of protozoa by sonication. The tube containing the protozoal suspension was immersed in an ultrasonic cleaning bath of average output 40 W and frequency 80 kHz at room temperature until no protozoa could be detected microscopically, as described by Coleman & Laurie (1977). The crude homogenate was centrifuged (5500 g, 20 min, 4 °C), the supernatant fluid was termed the broken-cell supernatant fluid and the pellet, after washing once by centrifuging in salt solution D, was the broken-cell pellet. When the protozoa were disrupted before incubation, the homogenate was centrifuged and the pellet was washed (× 3) in salt solution D (5500 g, 20 min, 4 °C) before plating out for the determination of radioactivity. This pellet was similar to the broken-cell pellet referred to above and contained any bacteria, polysaccharide granules and cellulose that had been present in the protozoa.

Preparation of [^{14}C]cellulose and [^{14}C]starch grains. These were prepared as described by Coleman *et al.* (1976) and Coleman (1969), respectively, and had specific activities of 2820 and 505 c.p.m. μg^{-1} , respectively. The dry α -cellulose was stored at 4 °C and phosphoric acid-regenerated cellulose was prepared from this as required.

Cellulase. This was assayed as described by Coleman *et al.* (1976) except that after incubation 0.1 ml of 1 % (w/v) haemoglobin was added to each tube before immersion in boiling water for 5 min. Each assay tube contained 2.5 to 9.0 μg [^{14}C]cellulose (7000 to 25000 c.p.m.) depending on the batch of phosphoric acid-regenerated cellulose used. As all batches had the same specific activity and as the amount of ^{14}C released during the assay was proportional to the amount of [^{14}C]cellulose added, it was possible to compare activities measured with different cellulose preparations. Under standard conditions cellulase activities were calculated on the basis that the amount of [^{14}C]cellulose added to each assay tube was 5000 c.p.m. Because the radioactivity released from [^{14}C]cellulose was not proportional to the amount of enzyme solution added, results of experiments on the comparison of the cellulase activities in different preparations from the same protozoal suspension are expressed relative to that in the broken-cell supernatant fluid by reference to a calibration curve such as that shown in Fig. 3. It is assumed that there is the same relationship between cellulose digested and enzyme concentration in all preparations.

Other procedures. Protozoa were fractionated as described by Coleman (1969). The number of protozoa was estimated as described by Coleman *et al.* (1976). Protein was determined by the method of Lowry *et al.* (1951). Cellulose was hydrolysed as described by Jermyn (1955) and the glucose content was determined by the method of Huggett & Nixon (1957). Volatile fatty acids were separated directly from acidified incubation media (after removal of particulate matter) by gas-liquid chromatography on a 1.8 m × 6 mm glass column packed with 20 % n eopentyl glycol adipate on 60/85 Universon B at 120 °C. Eluted compounds were identified by comparison of the retention times with those of known acids. The amounts of the fatty acids were calculated from retention time × peak height by reference to those of known amounts of the same compounds.

RESULTS

Survival of washed suspensions

The number of *E. maggii* in a washed suspension incubated anaerobically in the presence of ampicillin (1 mg ml $^{-1}$) declined to nil after 2 d in the absence of substrate but remained

Fig. 1. Survival of washed suspensions of *Eudiplodinium maggii* (235 ml^{-1}) incubated anaerobically with ampicillin (1 mg ml^{-1}) alone (Δ), or with ampicillin and the following additions (0.7 mg ml^{-1}): ●, cellulose; ○, cellulose and 10% (v/v) prepared fresh rumen fluid; □, rice starch; ■, rice starch and 10% (v/v) prepared fresh rumen fluid.

constant for 2 d in the presence of cellulose (Sigma, MN; 0.7 mg ml^{-1}) (Fig. 1). The further addition of 10% (v/v) prepared fresh rumen fluid (from which the protozoa had been removed; Coleman, 1958) increased the protozoal population density by 60% (100% in one experiment) after 2 d. At most times there were fewer protozoa in the presence of rice starch than cellulose suggesting that with protozoa grown *in vitro* on dried grass as the sole source of food, cellulose was the preferred substrate. Glucose or cellobiose (0.2%, w/v) did not improve survival over that in the absence of substrate.

Metabolism of intact protozoa

Cellulose. It was difficult to obtain an accurate estimate of the amount of [^{14}C]cellulose fermented because residual cellulose could not be completely separated from protozoa which also contained undigested cellulose and polysaccharide granules derived from cellulose. On incubation of *E. maggii* (10 500 organisms of isolate w grown *in vitro*) with [^{14}C]cellulose ($46.5 \mu\text{g}$) for 48 h in the presence of ampicillin (1 mg ml^{-1}), 34% of the ^{14}C not present in particulate form appeared as volatile fatty acids and 2% as non-volatile material in the medium. The remainder, which was not retained on the planchet in the presence of NaOH, was probably CO_2 . Of the ^{14}C found in the protozoal fraction, 1 to 5% (depending on the experiment) was in the broken-cell supernatant fluid and of this, 42% was in the residual protein (principally as alanine, aspartic acid and glutamic acid), 38% in the pool and 20% in the hot trichloroacetic acid (TCA)-soluble fraction. On further hydrolysis of this material (1 M-HCl, 100°C , 1 h), the products were glucose (94%) and a pentose, probably ribose (6%). As undigested cellulose was present in the broken-cell pellet, it was not possible to measure the incorporation of ^{14}C into fractions of this material. However, after hydrolysis by HCl of the material soluble in hot TCA, [^{14}C]glucose was the only product, suggesting that the ^{14}C was present as a glucose polysaccharide such as starch. To determine if the ^{14}C -labelled protein found in the broken-cell supernatant fluid was the product of bacterial action, the rate of synthesis was measured over 6 h in the presence and absence of chloramphenicol ($80 \mu\text{g ml}^{-1}$) (an inhibitor of bacterial protein synthesis), and was found to be 1.9 and $2.9 \text{ pg h}^{-1} \text{ protozoon}^{-1}$, respectively. This suggests that some of the protein in this fraction could have come from bacteria.

As well-fed protozoa produced volatile fatty acids from endogenous substrates ($45 \text{ pmol h}^{-1} \text{ protozoon}^{-1}$ over 24 h), it was difficult to determine the amount and proportions of

these produced from non-radioactive substrates. On addition of [^{14}C]cellulose or rice starch, there was no increase or a decrease (up to 35 %) in the rate of production over 24 h. On incubation of starved protozoa (100 000 organisms of isolate w grown *in vitro*) with cellulose or rice starch (4 mg) in the presence of ampicillin (1 mg ml $^{-1}$), the following amounts of short chain fatty acids were produced, respectively, in 24 h (in pmol h $^{-1}$ protozoon $^{-1}$, with production in the absence of substrate in parenthesis): acetic acid, 7.40, 9.26 (1.28); propionic acid, 0.60, 1.10 (0.17); butyric acid, 2.60, 2.72 (0.25). In the absence of substrate the protozoa were dead after 24 h.

Starch. On incubation of [^{14}C]starch grains (430 μg) with washed *E. maggii* (12 600 organisms of isolate w grown *in vitro*) for 5.5 h in the absence or presence of chloramphenicol (80 μg ml $^{-1}$), the respective distributions of ^{14}C were: 6 % and 6 % in the 'bacterial fraction' probably as free starch grains, 25 % and 17 % as volatile fatty acids in the medium, 2 % and 26 % as non-volatile material [as maltose (40 %) and glucose (60 %)] in the medium and 12 % and 8 % in the protozoa. The remaining material was probably CO_2 . Of the protozoal ^{14}C in the absence of antibiotic, 35 % was in the broken-cell supernatant fluid and of this, 64 % was in the pool, 20 % in the hot TCA-soluble fraction and 16 % in protein. This was equivalent to a synthesis of 41.6 pg h $^{-1}$ protozoon $^{-1}$. In the pellet fraction the rate was 157 pg h $^{-1}$ protozoon $^{-1}$. In the presence of chloramphenicol, protein was synthesized at rates of 14.6 and 39.4 pg h $^{-1}$ protozoon $^{-1}$ in the broken-cell supernatant fluid and pellet fractions, respectively. These results suggest that in the absence of antibiotic some of the protein in both fractions was bacterial or derived from bacteria.

Glucose. Although glucose is not normally present at high concentrations in the rumen, except just after feeding, it is the major product of the digestion of cellulose and starch and was taken up from the medium by *E. maggii* (isolate w grown *in vitro*) at a rate of 14 pmol h $^{-1}$ protozoon $^{-1}$ (measured over 3.5 h) at infinitely high glucose concentration (determined as described by Coleman, 1967). Of the ^{14}C recovered after uptake of 0.19 μmol [U- ^{14}C]glucose by 12 000 protozoa during 4 h from a 0.067 mM solution in the absence of antibiotic, 40 % was in the broken-cell supernatant fluid. This was distributed between the pool (75 %), hot TCA-soluble fraction (18 %) and protein (7 %). The incorporation into protein represents a conversion of glucose-carbon into protein of 20 pg h $^{-1}$ protozoon $^{-1}$. In the broken-cell pellet, 34 % of the ^{14}C was present in protein and 57 % in a glucose-containing polysaccharide. On disruption of the protozoa before incubation with [^{14}C]glucose, incorporation into the broken-cell pellet was increased by 120 %, and of the ^{14}C taken up, 42 % was in protein. As the uptake was sensitive to chloramphenicol (90 % decrease in incorporation in the presence of 80 μg ml $^{-1}$), this suggested that it was due to the action of bacteria in the pellet. With intact protozoa, incorporation into the hot TCA-soluble and protein fractions was decreased in the presence of chloramphenicol by 34 % and 87 %, respectively, in the broken-cell supernatant fluid and by 0 % and 44 % in the broken-cell pellet, suggesting that some of the protein was, or was derived from, bacterial protein. The distribution of amino acids in the protein was similar under all conditions with aspartic acid, glutamic acid and alanine being most heavily labelled and trace amounts of ^{14}C in glycine, arginine, lysine, proline and serine.

Cellulase

The rate of release of soluble ^{14}C -labelled compounds from phosphoric acid-regenerated α -[^{14}C]cellulose by protozoal broken-cell supernatant fluid declined progressively with time (Fig. 2). In this experiment 70 % of the cellulose was digested in 48 h but in others over 90 % digestion was obtained. The amount of ^{14}C released after any time was not proportional to the concentration of broken-cell supernatant fluid, the rate per unit volume of fluid decreasing with increasing concentration of fluid (Fig. 3). However, the amount of ^{14}C released was proportional to the amount of [^{14}C]cellulose added initially. The optimum pH for digestion was 5.7 to 6.2 (Fig. 4). At pH 6.0 the amount of ^{14}C released was slightly greater

Fig. 2. Release of ^{14}C from $[^{14}\text{C}]$ cellulose (14400 c.p.m.) by the broken-cell supernatant fluid from 7150 protozoa.

Fig. 3

Fig. 4

Fig. 3. Effect of the concentration of broken-cell supernatant fluid (1 ml equivalent to 79000 protozoa) on the release of ^{14}C from $[^{14}\text{C}]$ cellulose (9500 c.p.m.) during 5 h incubation.

Fig. 4. Effect of pH on the release of ^{14}C from $[^{14}\text{C}]$ cellulose (8000 c.p.m.) by the broken-cell supernatant fluid from 2200 protozoa during 6 h incubation in: ■, 0.1 M- $\beta\beta'$ -dimethylglutarate buffer; ●, 0.2 M- Na_2HPO_4 /0.1 M-citric acid buffer; ○, salt solution D (Coleman, 1972) adjusted to the pH value shown.

(15 to 21 %) in salt solution D than in phosphate/citrate buffer or $\beta\beta'$ -dimethylglutarate buffer (Fig. 4) although the addition of CaCl_2 or MgCl_2 (each at 1 or 10 mM) to the latter buffer was not stimulatory. Glucose was the major (over 90 %) digestion product unless cellobiose was added to the digestion mixture when, after 4 h, 60 % of the ^{14}C was present as cellobiose (for method of identification see Coleman, 1969).

In experiments made under standard conditions where less than 20 % of the $[^{14}\text{C}]$ cellulose was digested in 4 to 5 h, the rate of digestion by the enzyme in the broken-cell supernatant fluid was, with isolates co2 and 4L, $11.0 \pm 5.7 \text{ pg h}^{-1} \text{ protozoon}^{-1}$ ($n = 16$) and with isolate c, $28.4 \pm 8.1 \text{ pg h}^{-1} \text{ protozoon}^{-1}$ ($n = 4$). These are equivalent to 0.46 ± 0.24 and $3.3 \pm 0.9 \text{ } \mu\text{g cellulose digested h}^{-1} (\text{mg protein})^{-1}$, respectively.

Digestion of cellulose by disrupted protozoa. Disruption of protozoa markedly increased the amount of ^{14}C released from $[^{14}\text{C}]$ cellulose during short (5 h) incubations, probably due to the release of intracellular enzymes (Table 1). The supernatant fluid obtained after centrifuging the disrupted protozoa at low speeds (200 g, 20 s) was often more active than the starting homogenate, possibly due to the removal of cellulosic fragments that would compete with the $[^{14}\text{C}]$ cellulose for enzyme during the assay. Little activity (< 25 %) was

Table 1. *Cellulase activity of fractions of Eudiplodinium maggii grown in vivo*

Washed protozoa were disrupted by sonication in an ultrasonic cleaning bath (average output 40 W and frequency 80 kHz) at room temperature until no protozoa could be detected microscopically, and the homogenate was then centrifuged (200 g, 20 s). The resulting supernatant was centrifuged successively at two higher speeds. The pellet from each sedimentation was washed once and made up to the original volume with salt solution D containing 0.1 % cysteine hydrochloride, before assaying 0.1 ml of each fraction for cellulase activity. Activities are expressed relative to that of broken-cell supernatant fluid (taken as 100) – see Methods.

Fraction	Cellulase activity
Intact protozoa	10
Sonicated protozoa	90
Supernatant fluid (200 g, 20 s)	152
Supernatant fluid (5500 g, 20 min)	100
Pellet (5500 g, 20 min)	17
Supernatant fluid (114000 g, 60 min)	90
Pellet (114000 g, 60 min)	16

Table 2. *Effect of antibiotics on the cellulase activity in Eudiplodinium maggii*

Washed protozoa (1.26×10^6 grown *in vivo*) were incubated anaerobically for 22 h in salt solution D containing 0.1 % cysteine hydrochloride, with or without ampicillin (1 mg ml⁻¹) or chloramphenicol (60 µg ml⁻¹) and with or without cellulose (2.3 mg ml⁻¹). The protozoa were then disrupted by sonication (see Methods) and centrifuged (5500 g, 20 min) to produce the broken-cell supernatant fluid; the pellet after washing once was the broken-cell pellet. Cellulase activities are expressed relative to that of freshly prepared broken-cell supernatant fluid (taken as 100) – see Methods.

Incubation conditions		Cellulase activity in:	
Antibiotic	Cellulose added	Broken-cell supernatant fluid	Broken-cell pellet
No incubation		100	22
None	None	33	12
Ampicillin	None	36	8
Chloramphenicol	None	33	7
None	+	21	22
Ampicillin	+	27	29
Chloramphenicol	+	22	23

recovered in the pellet fractions after centrifuging at 5500 g or 114000 g showing that most of the cellulase was not particulate and that the activity of the initial homogenate was not due to intact bacteria.

Effect on protozoal cellulase of pre-incubation with antibiotics. As *E. maggii* contains bacteria (Hungate, 1966) it was possible that the cellulase liberated on breakage of the protozoa was of bacterial rather than protozoal origin. This was investigated by measuring the cellulase activity of the protozoa (1.26×10^6 , taken from the rumen) after incubation of the intact protozoa with or without antibiotics for 22 h *in vitro* (Table 2). In the absence of added substrate the activity of the broken-cell supernatant fluid decreased by 65 % with or without ampicillin (1 mg ml⁻¹) or chloramphenicol (60 µg ml⁻¹) whereas that of the pellet fractions decreased by 45 % with and 64 to 68 % without the antibiotics. This different sensitivity of the activity in the two fractions suggests that the enzyme in the supernatant fluid was of protozoal origin and that in the pellet of bacterial origin. On incubation of the protozoa in the presence of cellulose (2.3 mg ml⁻¹) the results were less clear, but there was little or no fall in the activity of the pellet fraction under any condition.

DISCUSSION

There is now considerable evidence that many of the larger Entodiniomorphid protozoa, e.g. *Polyplastron multivesiculatum*, *Enoploplastron trilocicatum*, *Diplodinium* spp. and *Eudiplodinium* spp., can utilize cellulose because these species can grow on cellulose-containing substrates in the absence of starch (see, for example, Hungate, 1942, 1943; Naga & El-Shazly, 1968; Coleman *et al.*, 1976). Washed suspensions have also been shown to degrade cellulose preparations (Hungate, 1943; Abou Akkada *et al.*, 1963; Coleman *et al.*, 1976) although Naga & El-Shazly (1968) found that whereas *Eudiplodinium* (? *Metadinium*) *medium* apparently utilized barley hay, cell-free extracts were inactive against cellulose. Although Hungate (1943) showed microscopically that *E. maggii* synthesized 'paraglycogen' from cellulose, the present paper is the first study on the incorporation of ^{14}C from [^{14}C]-cellulose by rumen protozoa. The results confirm that cellulose-carbon is incorporated into a glucose-containing polymer which is easily hydrolysed by acid and which is probably starch-like.

The fact that incubation with antibiotics has no effect on the level of cellulase in the cytoplasmic fraction (broken-cell supernatant fluid) confirms the suggestion of Hungate (1943) that the enzyme is not of bacterial origin although there may be a bacterial enzyme in the particulate fraction of the protozoa. Bacteria may be more important in the synthesis of protein from cellulose and starch. The presence of a chloramphenicol-sensitive protein synthesis in the cytoplasm suggests that bacteria in the protozoa synthesize protein from carbohydrate and are then digested by the protozoa with the liberation of amino acids that are synthesized into protein by the protozoa.

Although the experiments reported above were made with washed suspensions of *E. maggii*, which may not behave in the same way as growing organisms, the results can be used to obtain information on the importance of the protein synthesized from carbohydrate towards the total required by the protozoa for growth. As the protozoa have associated bacteria that are found after sonication in the broken-cell pellet, only incorporation into the broken-cell supernatant fluid will be considered. Each *E. maggii* organism (isolate w) grown in the rumen contained 24 ng protein in this fraction and would have to synthesize $1.0 \text{ ng protein h}^{-1}$ to divide once each day. As each protozoon synthesized (in the absence of antibiotic) $41.6 \text{ pg protein h}^{-1}$ from starch, 2.9 pg h^{-1} from cellulose and 20 pg h^{-1} from glucose (0.67 mM), the mean generation times of protozoa using these carbohydrates as sole source of protein would be 580, 8300 and 1200 h, respectively. It is probable that when growing on starch or cellulose, which are its natural food materials, *E. maggii* obtains the remainder of the amino acids from the digestion of engulfed bacteria or from free amino acids as found with other Entodiniomorphid protozoa (Coleman & Laurie, 1974).

The maximum rate of formation of short-chain fatty acids by *E. maggii* from endogenous substrate ($45 \text{ pmol h}^{-1} \text{ protozoon}^{-1}$) is equivalent to a rate of 135 mmol d^{-1} in a sheep rumen of 2.5 l fluid volume containing $50000 \text{ protozoa ml}^{-1}$. As a sheep produces a total of 4 mol d^{-1} (Leng, 1970), the production of short-chain fatty acids by this protozoon accounts for less than 3.5 % of the total. Although starch and cellulose are degraded to glucose their failure to stimulate the production of volatile fatty acids, except in protozoa that were almost dying of starvation, suggests that *E. maggii* like *Entodinium caudatum* (Coleman, 1969) possesses a mechanism for maintaining constant its intracellular glucose concentration and hence the rate at which it is metabolized.

I wish to thank Dr P. Kemp for inserting rumen cannulae into the sheep used to provide rumen fluid, Mr D. W. Grime for estimating fatty acids and D. C. Sandford and Y. S. Rutland for valuable technical assistance.

REFERENCES

- ABOU AKKADA, A. R., EADIE, J. M. & HOWARD, B. H. (1963). The carbohydrases of *Polyplastron multivesiculatum* (Dogiel & Fedorowa). *Biochemical Journal* **89**, 268–272.
- ABOU AKKADA, A. R., BARTLEY, E. E., BERUBE, R., FINA, L. R., MEYER, R. M., HENDRICKS, D. & JULIUS, F. (1968). Simple method to remove completely ciliate protozoa of adult ruminants. *Applied Microbiology* **16**, 1475–1477.
- COLEMAN, G. S. (1958). Maintenance of oligotrich protozoa from the sheep rumen *in vitro*. *Nature, London* **182**, 1104–1105.
- COLEMAN, G. S. (1967). The metabolism of free amino acids by the rumen ciliate *Entodinium caudatum*. *Journal of General Microbiology* **47**, 433–447.
- COLEMAN, G. S. (1969). The metabolism of starch, maltose, glucose and some other sugars by the rumen ciliate *Entodinium caudatum*. *Journal of General Microbiology* **57**, 303–332.
- COLEMAN, G. S. (1972). The metabolism of starch, glucose, amino acids, purines, pyrimidines and bacteria by the rumen ciliate *Entodinium simplex*. *Journal of General Microbiology* **71**, 117–131.
- COLEMAN, G. S. & LAURIE, J. I. (1974). The metabolism of starch, glucose, amino acids, purines, pyrimidines and bacteria by three *Epidinium* spp. isolated from the rumen. *Journal of General Microbiology* **85**, 244–256.
- COLEMAN, G. S. & LAURIE, J. I. (1977). The metabolism of starch, glucose, amino acids, purines, pyrimidines and bacteria by the rumen ciliate *Polyplastron multivesiculatum*. *Journal of General Microbiology* **98**, 29–37.
- COLEMAN, G. S., LAURIE, J. I., BAILEY, J. E. & HOLDGATE, S. A. (1976). The cultivation of cellulolytic protozoa isolated from the rumen. *Journal of General Microbiology* **95**, 144–150.
- HUGGETT, A. ST G. & NIXON, D. A. (1957). Use of glucose oxidase, peroxidase and *o*-dianisidine in determination of blood and urinary glucose. *Lancet* *ii*, 368–369.
- HUNGATE, R. E. (1942). The culture of *Eudiplodinium neglectum*, with experiments on the digestion of cellulose. *Biological Bulletin. Marine Biological Laboratory, Woods Hole, Massachusetts* **83**, 303–319.
- HUNGATE, R. E. (1943). Further experiments on the cellulose digestion by the protozoa in the rumen of cattle. *Biological Bulletin. Marine Biological Laboratory, Woods Hole, Massachusetts* **84**, 157–163.
- HUNGATE, R. E. (1966). *The Rumen and its Microbes*. New York: Academic Press.
- JERMYN, M. A. (1955). Cellulose and hemicelluloses. In *Modern Methods of Plant Analysis*, vol. 2, pp. 197–225. Edited by K. Paech and M. V. Tracey. Berlin: Springer-Verlag.
- LENG, R. A. (1970). Formation and production of volatile fatty acids in the rumen. In *Physiology of Digestion and Metabolism in the Ruminant*, pp. 406–421. Edited by A. T. Phillipson. Newcastle-upon-Tyne: Oriel Press.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- NAGA, M. A. & EL-SHAZLY, K. (1968). The metabolic characterization of the ciliate protozoon *Eudiplodinium medium* from the rumen of buffalo. *Journal of General Microbiology* **53**, 305–315.