The Metabolic Characterization of the Ciliate Protozoon *Eudiplodinium medium* from the Rumen of Buffalo

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SUMMARY

An oligotrich protozoon identified as *Eudiplodinium medium* was separated in pure culture and inoculated into the rumen of an isolated ciliate-free buffalo (*Bos bubalus* L.) calf to act as a continuous source of the organism. Analysis of the protozoa showed that a single organism contained on average 3.35 ± 0.003 μ g. protein; the carbohydrate fraction (49.1 % of dry matter) gave on hydrolysis 60.4 % glucose. Growth rate of the protozoa *in vitro* was dependent on food concentration. The number of protozoa was doubled in 2 hr at a substrate concentration of 0.23 mg./protozoon; at lower concentrations the doubling time was greater; at higher concentrations (0.32 mg./protozoon) the organisms burst.

Eudiplodinium medium did not metabolize simple sugars; it utilized starch, amylopectin, amylose and showed highest activity with hemicelluloses. Xylans were degraded faster than arabans; xylans gave relatively lower amounts of volatile acids than did arabans; acetic and butyric acids predominated, formic acid and traces of propionic and lactic acids were found but no succinic acid. No cellulose digestion took place during incubation for 12 hr; with longer periods of incubation with cellulose the organisms died. Ammonium salts, urea and amino acids were not used by the protozoa; proteolytic activity was observed towards gelatin, casein and gluten in descending order of activity. Cell-free extracts of *E. medium* showed activities towards carbohydrates as did whole organisms; xylanase activity was greatest. This enzyme was purified 28-fold; it had an optimum pH of 7.5 and an optimum temperature of 43°.

INTRODUCTION

The present work is concerned with the isolation of a ciliate oligotrich protozoon, *Eudiplodinium medium*, from the rumen of buffalo (*Bos bubalus* L.). Under similar conditions of feeding, the numbers of this ciliate were greater in buffalo than in cow. The metabolism of this organism was then studied.

METHODS

Isolation of Eudiplodinium medium. Unsuccessful attempts were made to isolate this oligotrich by fractional sedimentation from long columns filled with buffer (Oxford, 1951); the sedimented organisms were contaminated with *Entodinium*. *Isotricha* species were destroyed by adding mannose (Abou Akkada & Howard, 1960) to the medium. It was soon realized that Hungate medium (1942) was selective for Eudi-

plodium species. A strained rumen sample containing a complete protozoan population, obtained from a 4-month-old calf, was diluted 1/10 by Hungate medium. Rye grass in Hungate medium was replaced by barley fodder (harvested at 30 days), dried and added to a final concentration of 0.08 % (w/v). The medium was renewed twice daily by sucking off the upper half of the liquor. Within 7 days, types of protozoa other than *E. medium* and *E. neglectum* had disappeared. *E. medium* was predominant in most inocula; *E. neglectum* did not survive the competition and disappeared. The pure culture of *E. medium* was used for inoculating an isolated ciliate-free buffalo calf so that it only harboured this oligotrich, which reached a reasonable count within 10 days from inoculation. The calf was fed an early weaning concentrate mixture (Khoury, Ahmed & El-Shazly, 1967) and a green fodder *Lolium pereniale*.

Preparation of suspensions of washed protozoa. Rumen samples were withdrawn from the experimental calf before feeding and the protozoa separated and washed as recommended by Oxford (1951). Chloramphenicol (50 µg./ml.) was added to the last washings to decrease the number of bacteria; complete elimination of bacteria was not possible, but their activity was completely suppressed for a period of 18 to 22 hr after the addition of chloramphenicol, as judged by complete lack of glucose fermentation during this period, after which glucose fermentation was resumed, unless a further dose of chloramphenicol was added. All fermentations were done within 12 hr after adding chloramphenicol.

The counting technique. The *in vitro* growth rates were followed by counting protozoa in samples taken at different times. Since the number of protozoa in the cultures was small because of restricted sampling from the calf's rumen, an attempt was made to concentrate the protozoal content of a measured volume into one drop. The initial and final numbers of protozoa were counted in each treatment by withdrawing a 5 ml. sample from a known volume of the culture, under test by using a wide-mouthed graduated pipette. The pipette was left standing vertically for a few minutes until the protozoa contained in the sample sedimented to near the tip of the pipette. A small drop containing all the accumulated protozoa was then pipetted on to a slide and covered with a coverslip (2.3 × 5.1 cm.) so as to spread uniformly under it. Care was taken not to allow the formation of air bubbles. Counting was done at low power having a field area of 1 mm.². In each slide 30 fields were counted. Three slides were prepared from each sample. This counting technique was simple and gave reproducible results having a coefficient of variability of 6.94 % (ranging from 0.7 to 14 %). The slides were prepared in such a way as to have no more than 15–20 protozoa/field.

Metabolic activity. Washed protozoa in chloramphenicol + CO₂ + buffer were distributed in 10 ml. samples into test-tubes containing a previously weighed amount of substrate. In all incubations the final substrate concentration was 0.20 %. With each run an identical sample of protozoa-free supernatant fluid from the washed suspension was tested with a similar weight of substrate; this latter tube served as a control for detecting bacterial activity (Williams, Davis, Doetsch & Gutierrez, 1961). Bacterial numbers were greatly decreased by the washing used and by the chloramphenicol; this confirms the findings of Coleman (1962, 1967).

All incubations were done at 40° and lasted for 6 hr with soluble sugars and 12 hr with polysaccharides and proteins. After saturation with CO₂ the tubes were stoppered tightly with rubber bungs. After each run the viability of the protozoa was examined microscopically on a warm slide. At the end of each incubation period, the end pro-

ducts in the supernatant fluid were determined and expressed as end product/mg. protozoal-N/hr.

The mono- and di-saccharides were of AR grade (British Drug Houses Ltd.); the polysaccharides were given by Dr Abou Akkada; amino acids, urea, ammonium sulphate and proteins were of GR grade (Merck).

The preparation of cell-free extracts. Cell-free extracts of protozoa were prepared according to Abou Akkada, Eadie & Howard (1963) and kept under toluene in the refrigerator. It was checked for absence of bacteria by culturing in Coleman's media a, b, and c (1962) and found free.

Enzyme activities of cell-free extracts. One ml. citrate phosphate buffer, 0.05 g. substrate in 0.5 ml. buffer solution and 0.2 ml. of cell-free extract were incubated for 1 hr to test for enzyme activities. Incubations were made at different pH values to find the optimum pH values.

Methods of analysis. Reducing sugars were estimated according to Somogyi (1945) with copper sulphate and Nelson's (1944) arsenomolybdate reagents. Sugars were identified on paper chromatograms according to the method of Trevelyan, Procter & Harrison (1950). R_F values were determined for all mono- or di-saccharides used or expected to result from hydrolysis of polysaccharides.

Organic acids were separated for individual determination on silica gel columns according to Bulen, Warner & Burrell (1952) as modified by El-Shazly, Abou Akkada & Naga (1963).

Proteolytic activity was followed colorimetrically by using the ninhydrin reagent (Hawk, Oser & Summerson, 1953). A standard curve was plotted showing the relation between ninhydrin readings and nitrogen concentration of an amino acid mixture (casein acid-hydrolysate). Total N was determined by the micro-Kjeldahl technique (Chibnall, Rees & Williams, 1943).

Urea was estimated by using soybean meal (British Drug Houses Ltd.) as a source of urease. Ammonia was measured by the microdiffusion technique (Conway, 1957).

Analysis of the protozoa. An acetone-dried mass of washed protozoa was used for determination of the cell composition. Protein ($N \times 6.25$) was determined by the micro-Kjeldahl method. Lipids were extracted exhaustively with light petroleum (40° – 60°). For the determination of the carbohydrate fraction, proteins and polysaccharides were first dissolved in 30% NaOH (Hungate, 1963); the cellulose and non-cellulose fractions were determined in the alcohol precipitate according to Abou Akkada *et al.* (1963) and Abou Akkada & Howard (1960), respectively. The total sugar content was determined by the anthrone technique (Colvin, Attebery & Ivy, 1961). Glucose was estimated by the specific technique of Hyvariner & Nikkila (1962); this value was subtracted from the anthrone value to obtain the non-glucose sugar fraction.

The amino acid pattern of the protozoal protein was examined by using two-dimensional paper chromatography in phenol–0.3% NH_3 and *n*-butanol+acetic acid+water (40+10+50 by vol.). Acid hydrolysis was done in sealed tubes with a 6 N-HCl for 22–24 hr. Alkaline hydrolysis was done by boiling in saturated $Ba(OH)_2$ solution for 2 hr.

RESULTS AND DISCUSSION

Eudiplodinium medium (Awerinzew & Mutafova, 1914) was photographed and described by Hungate (1966) as 'one of the largest rumen protozoa...its macronucleus is depressed at two points on the dorsal side adjacent to the contractile vacuoles (Rees, 1931), it has two skeletal plates'. These morphological characteristics are typical of the species studied in the present work. A photograph of an iodine-stained cell is shown in Pl. I, fig. 1. Since it was difficult to obtain a clear photograph of the nucleus, the haematoxylin (Heidenhain) stain was applied to a specimen according to Gurr (1953); the stained nuclei are shown in Pl. I, fig. 2.

The proximate cell composition of Eudiplodinium medium

The proximate analysis of a mass of protozoa of *E. medium* showed the following composition (% of dry matter): crude protein, 42.3; carbohydrates, 49.1; light petroleum extract, 7.8; ash 0.8. The protein content of this organism may appear somewhat high when compared with that of a mixed protozoal population (McNaught, Owen, Henry & Kon, 1954). The authors ascribed the low protein content of their mixed protozoal preparation to its high content of stored polysaccharides; their protozoa had been separated from rumen samples obtained 2 hr after feeding. In the present study, rumen samples were obtained before the morning ration, about 15 hr after the last meal.

The carbohydrate fraction of *E. medium* gave on hydrolysis 60.4 % glucose: the remaining 39.6 might have included pentoses from the nucleic acids.

The amino acid pattern of the protein fraction of this organism showed that glutamic and aspartic acids were predominant. The quantitative amino acid analyses of Weller (1957) and Purser & Buechler (1966) showed these two acids to account for about 25 % of the protozoal protein. The chromatograms from the acid and alkaline hydrolyses of some organisms showed the presence of all the essential amino acids. Tryptophan was not detected on the chromatograms.

Three suspensions containing about 2600, 600 and 300 of *Eudiplodinium* organisms/ml. were found to contain 8.71, 2.04 and 0.98 mg. protein/ml. respectively; indicating that the protozoa contained 3.35 ± 0.003 μ g. protein/organism.

Growth rates of Eudiplodinium medium

Hungate (1942) obtained a culture containing over 1000 organisms of *Eudiplodinium neglectum*/ml. at 0.08 % (w/v) feed concentration; increasing the substrate concentration favoured protozoal growth but the culture medium needed more frequent renewal. Cultures containing fewer protozoa and the same substrate concentration did not survive; it was suggested that this was because of the presence of toxic metabolic products. In the present cultures of *E. medium* the number of organisms never exceeded 50 to 100/ml. with a feed concentration (barley hay) of 0.08 % (w/v). Even with as few protozoa as 5 to 10/ml. there was good growth, but only when the feed concentration did not exceed 0.23 mg./protozoa.

Table 1 shows the relationship between substrate concentration (barley hay) in mg./protozoa and the growth of *E. medium*. A concentration of 0.23 mg. feed/protozoon was most favourable for growth. The initial number of protozoa was doubled within 2 hr, as compared with 14 and 7 hr for the concentration of 0.08 and 0.16 mg.

feed/protozoa, respectively. Higher concentrations (0.32 mg./protozoa) caused rupture of the organisms as observed microscopically. It is possible that the higher substrate concentrations per *E. neglectum* was the reason for their non-survival in the cultures of lower population densities prepared by Hungate (1942). On the other hand the longer generation time (48 hr) reported by Hungate (1942) was probably due to the much lower concentration of feed/protozoon (0.0007 mg.) used in his culture. The different generation times reported for different protozoal species (Gutierrez, 1955, 1959; Warner, 1962; Coleman, 1962; Mah, 1962) may have reflected limitations due to substrate concentration/organism rather than a character of the species.

Table 1. *The relationship between substrate concentration (barley hay in Hungate medium) (mg./protozoon) and the concentration of Eudiplodinium medium (organisms/ml.)*

Time from start (hr)	Substrate concentration (mg./protozoon)			
	0.08	0.16 Protozoa (no./ml.)*	0.23	0.32
0	10.0	5.0	4.4	2.5
1	10.0	4.8	4.7	3.3
2	11.4	5.9	9.0	2.8
3	12.2	7.6	13.9	2.5
4	13.0	8.4	15.7	—
5	13.8	9.0	17.9	—
6	14.1	9.6	20.6	—
7	14.9	9.9	20.7	1.8
11	18.3	5.7	11.2	0.7
14	20.2	—	—	—

* Averages of three determinations (see text).

Table 2. *Eudiplodinium medium: the exhaustion of stored polysaccharides of protozoa in presence of glucose*

Time (hr)	Glucose in medium		Protozoal polysaccharides	
	µg./ml.	% of initial	µg./ml.	% of initial
0	179.0	100.0	260.0	100.0
4	184.0	102.8	182.5	70.0
7	193.0	108.0	135.0	52.0

The metabolic activities of Eudiplodinium medium

Eudiplodinium medium did not utilize glucose, fructose, mannose, galactose, xylose, arabinose, galacturonate, maltose, sucrose, lactose, cellobiose or xylobiose. The protozoa were alive and showed active movement at the end of the incubation period. It was observed that the sugar concentration in the supernatant fluid after incubation had increased; this must have come from stored polysaccharide in the organisms (Table 2). The protozoa became transparent after 6 hr incubation with the soluble sugars; this is a sign of exhaustion of reserve material.

Eudiplodinium medium did not hydrolyse pectin, pectate, inulin, levan. Di- and poly-saccharides were run on paper chromatograms before and after incubation with *E. medium*; no new spots were observed after incubation. The ability of *E. medium* to engulf cellulose particles was observed microscopically, but no apparent utilization

could be demonstrated. No sugars were released into the medium above that of the endogenous values, because of cellulose ingestion at 12 hr intervals. Incubations with cellulose for longer periods resulted in death of the organisms. Volatile fatty acid yield was somewhat higher (0.079 μ -equiv./mg. protozoal-N/hr) in the cellulose incubations than in the controls. This might have been due to increased endogenous metabolism in the process of ingestion or to the presence of impurities (hemicellulose) in the cellulose preparation. Westphal (1934) observed the same species to ingest cellulose with no resulting deposition of polysaccharide reserves.

Table 3. *Eudiplodinium medium*: the soluble end-products of different polysaccharides fermented and the proportions (%) of volatile fatty acids (VFA) produced, expressed as end-product/mg. protozoal-N/hr

Polysaccharide	Reducing sugar produced above endogenous (μ g./mg. N/hr)	Total VFA produced above endogenous (μ -equiv./mg. N/hr)	Lactic acid* produced above endogenous (μ -equiv./mg. N/hr)	Proportions of VFA			
				Formic acid	Acetic acid	Propionic acid	Butyric acid
Rice starch	0.22 $\pm$ 0.062	0.43 $\pm$ 0.031	0.06	24.53 25.75	42.72 43.34	4.27 3.67	28.48 27.24
Amylose	0.58 $\pm$ 0.081	0.38 $\pm$ 0.040	—†	—	—	—	—
Amylopectin	0.96 $\pm$ 0.010	0.26 $\pm$ 0.040	—	—	—	—	—
Hemicellulose‡	0.64 $\pm$ 0.007	0.34 $\pm$ 0.036	0.05	18.41 16.20	54.74 59.42	3.48 3.33	23.37 21.05
Xylan	1.65 $\pm$ 0.097	0.02 $\pm$ 0.001	0.07	33.75 32.37	46.22 45.96	3.52 3.74	15.51 17.93
Araban	0.06 $\pm$ 0.003	0.34 $\pm$ 0.079	—	—	—	—	—
Cellulose	0.00	0.08 $\pm$ 0.016	—	—	—	—	—
Endogenous	0.01 $\pm$ 0.004	0.23 $\pm$ 0.060	0.05	12.85 11.12	57.66 56.67	4.29 4.25	25.20 27.20

* = one determination. † = not determined. ‡ = purified bran cellulose.

Table 3 gives the products of metabolism of rice starch, amylose, amylopectin, hemicellulose, xylan, araban and cellulose. Starch and hemicellulose are the major sources of energy for this organism. Glucose and maltose were detected on chromatograms prepared from starch, amylose and amylopectin incubations. Xylose, arabinose or both were detected on paper chromatograms prepared from xylan and hemicellulose fermentations respectively.

Eudiplodinium medium is like other protozoa in the general pattern of end products, producing traces of propionic acid and much acetic and butyric acids (Howard, 1963; Hungate, 1966). It also produces relatively large amounts of formic acid which seem to exceed that produced by any other rumen protozoa. Holotrichs do not produce formic acid (Heald & Oxford, 1953; Gutierrez, 1955; Howard, 1959); *Epidinium ecaudatum* (Gutierrez & Davis, 1962) and *Entodinium caudatum* (Abou Akkada & Howard, 1960) produce little formic acid. Succinic acid was not found among the fermentation products of *E. medium* and only a little lactic acid (Table 3).

Xylan was hydrolysed mainly to xylose which was not further degraded appreciably to volatile fatty acids. This could be an example of the phenomenon of hydrolysis of a polymer without utilization of the products which was reported by Abou Akkada & Howard (1961), who found that holotrichs hydrolysed pectic substances without utilizing the hydrolysis products. Dehority (1965) found that three strains of rumen

cellulolytic bacteria degraded and utilized xylan, while five other organisms, particularly *Bacteroides succinogenes*, degraded it. The hydrolysis of xylan by *E. medium* may have significance for the growth of rumen bacteria. Xylose favours protein synthesis (Hendrickx & Martin, 1963), an effect in this respect only surpassed by arabinose. In the present work xylose was not fermented and accumulated, arabinose (or perhaps the biose, as arabinose given externally is apparently not fermented) is fermented as fast as it is formed by hydrolysis of araban and does not accumulate.

Nitrogen metabolism

Urea and ammonium sulphate, in concentrations similar to that of artificial saliva (McDougall, 1948), were not utilized by *E. medium* even in the presence of amylopectin (0.2 %). A mixture of 20 amino acids (in concentration as recommended by Quinn, Burroughs & Christiansen, 1962) was not taken up or catabolized by this organism; no ammonia was produced when the organisms were incubated with the amino acid mixture for 6 hr. Abou Akkada & Howard (1962) did not detect any utilization or degradation of Amino acids by *E. caudatum* by using similar techniques. Coleman & Hall (1966) and Coleman (1967), using ^{14}C -labelled amino acids, provided evidence of a slow uptake of amino acids by *E. caudatum*; some volatile fatty acids were found but not ammonia.

Table 4. *Eudiplodinium medium*: the end-products of different proteins with and without energy source (amylopectin)

Protein substrate	NH_3 produced above the endo- genous ($\mu\text{g. NH}_3$ - N/mg. protein-N/ hr)	Amino acids pro- duced above endo- genous ($\mu\text{g. amino-}$ N/mg. protein-N/ hr)
Endogenous	0.48	19.62
Endogenous + amylopectin	—	6.65
Casein alone	—	26.00
Casein + amylopectin	—	45.00
Ground casein + amylopectin	—	14.32
Gluten + amylopectin	—	17.00
Ground gluten + amylopectin	—	11.09
Gelatin alone	—	39.21
Gelatin + amylopectin	—	58.36

Eudiplodinium medium produced ammonia and amino acids as end-products of endogenous N metabolism; the presence of amylopectin increased metabolic activity as indicated by higher amino acid production (Table 4). The addition of cysteine HCl (2 %) favoured proteolysis and was used in all experiments. Gelatin showed the highest rate of hydrolysis followed by casein. Gluten was the least hydrolysable (Table 4). It appeared that the rate of protein breakdown by *E. medium* ran parallel with the solubility of the protein. The addition of amylopectin to the protein increased proteolysis significantly. However, the proteolytic power of *E. medium* was much lower than that of *E. caudatum* (Abou Akkada & Howard, 1962). When casein and gluten were finely ground, proteolysis was considerably decreased (Table 4).

Activity of cell-free extracts of Eudiplodinium medium

Cell-free extracts of holotrichs were shown to exhibit activities corresponding to those of the whole living protozoa (Howard, 1959). Howard (1963) reported that the holotrich outer membrane was permeable, while that of oligotrichs was not. The cell-free extracts of oligotrichs seem to lack this parallelism in activity with the whole protozoa. Sugden (1953) and Howard (1963) reported that oligotrichs fermented only the insoluble particulated polysaccharides, and did not utilize soluble carbohydrates. The cell-free extracts of oligotrichs exhibited enzymic activities against soluble disaccharides (maltose, sucrose, cellobiose, xylobiose; Abou Akkada & Howard, 1960, 1962; Bailey & Clarke, 1963). On the other hand, there were enzymes detected in living protozoa which were not found in the corresponding cell-free extracts (Bailey & Howard, 1962; Bailey & Clarke, 1963). The cell-free extract of *E. medium* showed no proteinase activity which was observed with the living protozoa. Maltose and xylobiose were hydrolysed by the cell-free extracts of *E. medium* to a very small extent (about 8 %).

Table 5. *Eudiplodinium medium* cell-free extract: the specific activity of xylanase in the different ammonium sulphate precipitate fractions of the cell-free extract

The incubations lasted for 30 min. at 40°. Incubation mixture was: 2 mg. xylan added as 0.2 ml of 1% solution + 0.2 ml. cell-free extract and phosphate + citrate buffer (pH 7).

Crude cell-free extract	Ammonium sulphate (%) precipitate					
	10	20	30	40	50	60
	Xylose products ($\mu\text{g.}/\text{mg. N/min.}$)					
33.4	20.9	19.0	218.0	634.2	938.3	389.0

Figure 1 shows the relationship between pH value and xylanase, 1-4- α -glucuronidase, 1-6- α -glucosidase and arabinase activities, when tested under the same conditions. The activities in the cell-free extract of *E. medium* followed the same trend as the activities of the living protozoa (Table 3). The enzymes concerned with hemicellulose degradation (xylanase, arabinase) had different pH optima (7.5 and 5.5, respectively). The amylases had an optimum pH about 6.

Partial purification of the xylanase was effected by using different concentrations of saturated ammonium sulphate. Table 5 shows xylanase specific activity ($\mu\text{g.}$ xylose produced/ $\mu\text{g.}$ N/min.) of the crude cell-free extract and in the different ammonium sulphate precipitated fractions. The highest specific activity was found in the 50% ammonium sulphate precipitate which showed a 28.7-fold purification. Optimum pH values and temperature values obtained with the crude preparation were similar to those obtained with the purified preparation (Fig. 1, 2).

Xylanase activity of rumen liquor (Sorensen, 1955), rumen bacteria (Howard, Jones & Purdom, 1960) and of *Fusarium roseum* (Gascoigne & Gascoigne, 1960) have been studied; their respective pH values were found to be 6.3, 5.6 and 6.3. Three attempts were made to determine Michaelis constant of the enzyme xylanase from *E. medium*. Solubilized xylan (treated with hot 0.1 N-HCl) was used; the results were not reproducible. Howard *et al.* (1960) found that xylanase activity of rumen bacteria

Fig. 1. The pH-activity relationship of enzymes in cell-free extract of *Eudiplodinium medium*. Incubations at 40° for 1 hr. Incubation mixture: 2 mg. substrate added as in 0.2 ml. cell-free extract (crude); 0.6 ml. phosphate + citrate buffer of different pH values.

Fig. 2. *Eudiplodinium medium* cell-free extract. The temperature-activity relationship of the enzyme xylanase. Incubations for 1 hr at different temperatures. Incubation mixtures consisted of: 2 mg. xylan added as 0.2 ml. of a 1% solution; 0.2 ml. cell-free extract; 0.6 ml. phosphate + citrate buffer (pH 7).

decreased sharply as the degree of polymerization of the xylan was decreased, and reported that 'the reducing power of the enzyme + pentosan mixture became constant at a value suggesting that the pentosan had been broken down to oligosaccharides with degree of polymerization of about 12'.

In the present work when the prepared soluble xylan fraction was completely hydrolysed, it had a reducing power 13 times as great as before hydrolysis, suggesting a degree of polymerization of 13 units for the solubilized xylan fraction. From this point of view, the short-chain xylan preparation was not expected to respond as native xylan to the enzyme action. The enzyme action on this substrate ceased on hydrolysing 64 % of its structure.

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Fig. 1

Fig. 2

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EXPLANATION OF PLATE

Fig. 1. *Eudiplodinium medium* from the rumen of buffalo stained with iodine. $\times 160$.

Fig. 2. *Eudiplodinium medium* from the rumen of buffalo. Stained with haematoxylin to show the macronucleus. $\times 99$.