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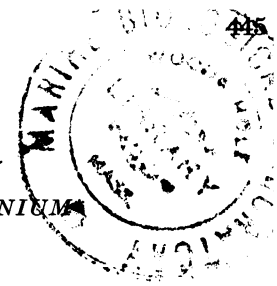

The Biochemistry of Rumen Protozoa

3. THE CARBOHYDRATE METABOLISM OF *ENTODINIUM*

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The rumens of sheep and cattle commonly contain ciliate Protozoa belonging to numerous genera. The biochemical study of these organisms has been hampered in the past by the difficulty of separating the different types from bacteria, and from one another, in sufficient quantity. The conventional bacterial pure-culture techniques have not so far proved applicable to these organisms, and investigators have therefore had to rely mainly upon alterations to the ruminant's diet, and various manipulations of the rumen liquor *in vitro*, in attempts to obtain suspensions of individual types of Protozoa. A technique of considerable value in this field was originated by Eadie & Oxford (1957), who found that many species of Protozoa could be eliminated from the sheep rumen by emptying and thorough washing of that organ. In a number of sheep treated in this way, only the smaller species of the oligotrich ciliate *Entodinium* survived the emptying procedure. It was further found (J. M. Eadie, personal communication, 1958) that when the diet of a treated sheep was altered by including a moderate amount of starchy foods, the numbers of entodinia present in the rumen increased to such an extent that suspensions of mixed *Entodinium* species, free from other Protozoa, and almost free from bacteria, could readily be prepared from the rumen liquor. Because *Entodinium* can, under many feeding régimes, be the predominant protozoal genus in the rumen, and because the biochemistry of these organisms has not been studied previously, the present investigation was undertaken. Some of our observations have already been reported briefly (Abou Akkada, Hobson & Howard, 1959).

MATERIALS AND METHODS

Sheep as source of entodinia. The single sheep (no. 105) which served throughout these experiments had previously had its rumen fauna partially removed by the method of Eadie & Oxford (1957), and thereafter was kept isolated from other ruminants. Before the present work was commenced it had been inoculated with *Dasytricha ruminantium*, but during our experiments only very small numbers of this organism were present. In addition to a feed of hay (450 g.) morning and afternoon, the sheep was offered daily 450 g. of a concentrates ration, approximately three-quarters of which consisted of ground maize and crushed oats.

Preparation of protozoal suspensions. Samples of rumen liquor were withdrawn 3 hr. after the concentrates meal, strained through gauze and mixed in a separating funnel with an equal volume of the bicarbonate buffer solution described below (cf. Oxford, 1958). All manipulations and incubation of protozoa were carried out at 38°. During incubation for 1 hr. the entodinia settled to the bottom of the funnel as a grey–white layer, which was run off into boiling tubes containing the buffer solution. From this stage onwards the buffer solutions used always contained chloramphenicol (50 μg./ml.) to suppress bacteria. After threefold washing by decantation, the protozoa formed a clean white layer in the bottom of the tubes. Microscopic examination of the protozoa, both living and after staining with iodine, showed them to be very active and filled with grains of plant starch at this stage. The small numbers of *D. ruminantium* in the suspensions were killed by incubating overnight in buffer solution containing 0·6% of mannose. After washing out the mannose by three changes of buffer solution the suspensions consisted almost wholly of *Entodinium caudatum*, with a very few small organisms similar to *Entodinium simplex*. Neither chloramphenicol nor mannose appeared to harm the entodinia. The suspensions could be kept alive for 3 days by changing the buffer–chloramphenicol solution every 12 hr. At the end of this period the cells were almost empty of starch.

Buffer solutions. The solutions used were based on that of Coleman (1958). The one normally used contained (in 1 l.); K_2HPO_4, 6·3 g.; KH_2PO_4, 5·0 g.; $NaHCO_3$, 1 g.; NaCl, 0·6 g.; $MgSO_4,7H_2O$, 0·1 g.; $CaCl_2,6H_2O$, 0·09 g.; Na_2S, $9H_2O$, 0·2 g. The pH was 6·9–7·0. In experiments which required the analysis of fermentation gases, the $NaHCO_3$ was replaced by sodium acetate (0·75 g./l.).

Materials. These were the same as those used by Howard (1959*a*, *b*). Amylose, amylopectin, β-dextrin and maltotriose were given by Dr P. N. Hobson. Chloramphenicol was B.P. quality, manufactured by Parke, Davis and Co. Ltd., Hounslow.

Bacterial examination of protozoal suspensions. Portions of protozoal suspensions were inoculated into the semi-solid medium of Hobson, Mann & Oxford (1958), in which soluble starch replaced the sodium lactate.

Fermentation procedures. (*a*) Endogenous fermentation. Sufficient washed protozoa were placed in 100 ml. conical flasks to just cover the bottom. This usually required the protozoa from 250 ml. of rumen liquor/flask. The flasks

* Part 2: Howard (1959*b*).

were immediately filled almost to the top with buffer–chloramphenicol solution, gassed for 3 min. with $N_2 + CO_2$ (95:5) and sealed with rubber stoppers carrying Bunsen valves. Samples for analysis were removed at intervals during incubation, after mixing the contents of the flasks by gentle swirling.

(*b*) Incubation with soluble sugars. A suspension of protozoa which had been starved for 36 hr. was divided equally between two flasks, which were treated as described above, except that to one flask the substrate was added to a final concentration of 0·2%. For analysis the samples of suspension were centrifuged, and the carbohydrate in the supernatant was determined by the anthrone method (Trevelyan & Harrison, 1952).

(*c*) Analysis of gases formed during endogenous fermentation. A suspension (200 ml.) of washed entodinia in the acetate buffer–chloramphenicol was placed in a 500 ml. flask immersed in a water bath at 38°. A slowly flowing stream of N_2, which had previously been bubbled successively through sodium pyrogallate and water, was delivered below the surface of the suspension. The determination of CO_2, H_2 and hydrocarbons in the emergent gases was carried out as described by Heald & Oxford (1953), except that the addition of O_2 to the gases before passing over the heated CuO was omitted. The fermentation was allowed to proceed for 6 hr., after which time 4 N-H_2SO_4 (2 ml.) was added to the suspension to kill the protozoa and release bound CO_2. The gas flow was continued for 1 hr. after addition of the acid. The weight of CO_2 found in the absorption tube was corrected for the small amount found in a blank experiment when buffer solution without protozoa was used.

Manometric methods. The methods for measuring the effect of protozoa on added carbohydrates were as described by Howard (1959*a*). In other experiments samples were taken from a suspension of entodinia at intervals during a 48 hr. incubation period and tested for rate of endogenous fermentation. The gaseous products of fermentation were also measured manometrically, by the method of Elsden & Lewis (1953). Warburg double-side-arm cups were used, containing protozoal suspension in acetate buffer (2 ml.) under an atmosphere of N_2, with 2 N-NaOH (0·2 ml.) absorbed in filter paper in one side arm, and 4 N-H_2SO_4 (0·4 ml.) in the other.

Preparation of cell-free extracts. Washed protozoa, freed from as much supernatant buffer solution as possible, were frozen and stored at −20°. When required, the frozen mass was thawed by incubating at 38° for 1 hr. and the suspension was mixed in a mortar with 3 vol. of carborundum powder (4F mesh, Griffin and George Ltd., Wembley, Middlesex). The stiff paste was ground by hand for 1 hr., transferred with a little water to centrifuge tubes and centrifuged at 20 000 *g* for 30 min. at 0°. After dialysing and centrifuging again (Howard, 1959*b*), the supernatant yielded a clear solution, which was stored at 1° under toluene. About 15 ml. of solution, containing approx. 180 μg. of protein N/ml., was obtained from the entodinia from 1 l. of rumen liquor.

Tests for carbohydrase activity. Qualitative tests were made as described by Howard (1959*b*). In addition, a chromatographic solvent, consisting of the top layer of benzene–pyridine–butanol–water mixture (1:3:5:3, by vol.) was used in examining amylose hydrolysates for maltotriose. In quantitative experiments with amylase, the procedure of Hobson & MacPherson (1952) was followed. To measure maltase activity, tubes containing maltose (100 μg.), 0·1 M-sodium citrate buffer (0·3 ml.) and suitably diluted enzyme (0·5 ml.) in a total volume of 1 ml. were placed in a water bath at 38° for 3 hr. The reducing power of the mixtures was measured by Nelson's (1944) method, and the extent of hydrolysis of maltose was calculated from the reducing power of maltose and glucose determined at the same time.

The separation of the amylase and maltase activities in the extracts was attempted by the method of Hockenhull & Herbert (1945). An ice-cold mixture of enzyme solution (5 ml.), Na_2SO_4 (50 mg.) and ethanol (5 ml.) was shaken for 2 hr. with potato starch [1 ml. of a 12·5% (w/v) suspension in 50% ethanol]. The starch was subsequently washed twice with 10 ml. of 0·067 M-phosphate buffer (KH_2PO_4–Na_2HPO_4), pH 5·8. The aqueous ethanol and phosphate solutions were dialysed and their amylase and maltase activities were determined.

Analytical methods. The starch in the protozoal cells was determined by an adaptation of the methods of Pirt & Whelan (1951) and Trevelyan & Harrison (1952). A portion (2 ml.) of the well-mixed suspension of protozoa was centrifuged at 600 *g* for 20 min. and the clear supernatant was decanted. In cases where soluble sugars had been added to the suspension, the cells at this stage were washed once with water (1 ml.). The cells were suspended in 0·25 N-NaOH (4 ml.) and the tubes were heated in boiling water for 3 min. After being centrifuged as before, a portion of the clear supernatant was suitably diluted and the total glucose present was determined by the anthrone method. Chromatographic examination of an acid hydrolysate (2 N-H_2SO_4 for 1 hr. at 100°) of the alkaline extract showed that no sugars other than glucose were present.

Volatile fatty acids, except formic acid, were detected on paper chromatograms by the method of Elsden & Lewis (1953); formic acid was detected by the ninhydrin method of Burness & King (1958). Quantitative determination of volatile fatty acids, lactic acid, total N and protein N was carried out as described by Howard (1959*a*). Dry matter in the suspensions was determined by drying them for 5 hr. at 105°, and correcting the weight of residue for the weight of salts remaining after drying an equal volume of buffer solution.

RESULTS

As prepared for use in fermentation experiments, the suspensions consisted almost wholly of *E. caudatum*, actively motile and well filled with grains of plant starch. In three of these suspensions, the protozoal dry matter was 11, 20 and 31 mg./ml. respectively. The protein content (total N × 6·25) was 23–26% (mean 25%) and the starch content (as glucose) 59–68% (mean 64%) of the protozoal dry matter. No bacteria able to grow in the semi-solid starch medium were ever observed when inoculations were made from the chloramphenicol-treated suspensions.

Endogenous fermentation by entodinia. When the suspensions were incubated in the bicarbonate buffer–chloramphenicol the organisms remained active for more than 48 hr., during which time the

starch in the cells largely disappeared, as judged by the iodine reaction. These microscopic observations accord with the quantitative results shown in Fig. 1. During incubation for 24 and 48 hr., 61 and 92% respectively of the starch originally present disappeared. The dwindling metabolic activity of the protozoa in the aging suspension, suggested by the shape of the curve in Fig. 1, is also illustrated by the manometric results (Fig. 2). Gas production by suspensions of all ages tested was linear during the first 30 min. of the manometric experiments. The rate of gas production, expressed as μl. of carbon dioxide/hr./0·1 mg. of N, dropped by almost a half during the first 24 hr. in the life of a suspension. This rate seemed remarkably constant between suspensions prepared at different times.

The products formed by the entodinia during the fermentation of ingested starch included volatile fatty acids, lactic acid, carbon dioxide and hydrogen. It was not possible to determine all of these products in the same suspension; the gaseous products on the one hand, and the volatile fatty acids and lactic acid on the other, had to be determined in separate experiments, as shown in Table 1. The volatile fatty acids, upon chromatographic analysis, were shown to consist of formic acid, acetic acid, propionic acid and butyric acid only, with no trace of any higher acid (Table 2). The acetic acid and butyric acid together accounted for 82 and 89% of the total volatile fatty acids in Expts. 1 and 2 respectively.

Effect of entodinia on added substrates. The gas production during incubation of 24 hr. old suspensions in the Warburg apparatus with glucose, maltose or rice-starch grains for 1 hr. was identical with that of control portions of suspensions incubated without added substrates. Microscopic examination of iodine-stained drops of the suspensions at the end of the run suggested that no conversion of soluble sugar into storage starch had occurred. Most of the starch grains, however, had been swallowed by the protozoa. In other experiments duplicate portions of protozoal suspensions were incubated with and without added soluble carbohydrate. Frequent samples were taken, and the starch content of the cells and the carbohydrate content of the solution were measured. The results of an experiment with glucose are shown in Fig. 3; similar results were obtained by the use of maltose, cellobiose, sucrose and soluble starch.

To a suspension of protozoa 36 hr. old, granular rice starch was added (4 mg./ml.), and the incubation continued for 12 hr. more. The endogenous fermentation rate, measured manometrically, had then risen above the value found just before addition of the starch (Fig. 2).

Carbohydrases in cell-free extracts of entodinia. The extracts of the protozoal cells had no

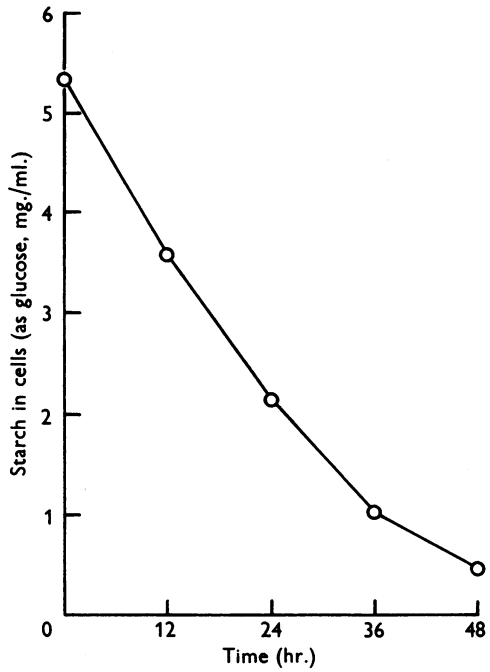

Fig. 1. Fermentation of ingested starch by suspensions of *E. caudatum*. For conditions of incubation see text.

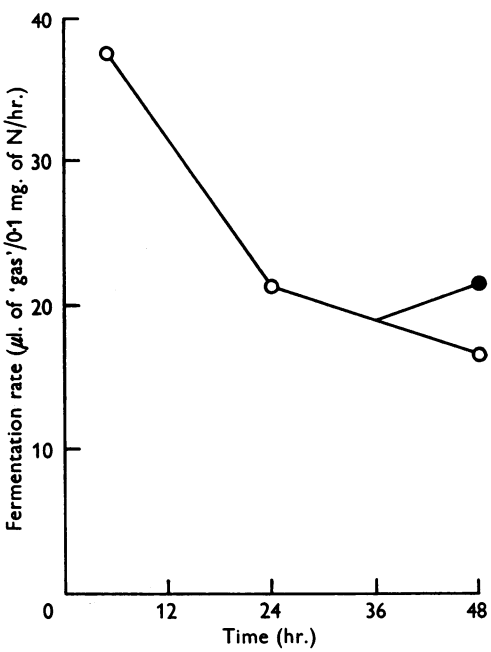

Fig. 2. Changes in the fermentation rate of suspensions of *E. caudatum* with age. ○, Fermentation rate of starved protozoa; ●, fermentation rate 12 hr. after adding granular rice starch (0·2%). For conditions see text.

Table 1. *Products of fermentation of ingested plant starch grains by suspensions of* Entodinium *species*

Results are expressed as μmoles of product from the fermentation of 100 μmoles of starch, as glucose. Experimental conditions: A1, 48 hr. and A2, 24 hr., both in bicarbonate buffer; B, 1 hr. in acetate buffer in Warburg apparatus; C, 6 hr. in acetate buffer, in N_2 stream.

Expt. no.	Volatile fatty acids	Lactic acid	CO_2	H_2
A1	156	4·42	—	—
A2	141	3·70	—	—
B1	—	—	107	100
B2	—	—	98	100
B3	—	—	104	83
B4	—	—	103	93
C1	—	—	120	122
C2	—	—	125	137

Table 2. *Volatile fatty acids produced in starch fermentation by suspensions of* Entodinium

Results are expressed as molar percentage of total volatile fatty acids.

Expt. no.	Formic acid	Acetic acid	Propionic acid	Butyric acid
A1	5	35	13·5	46·5
A2	4	34	7	55

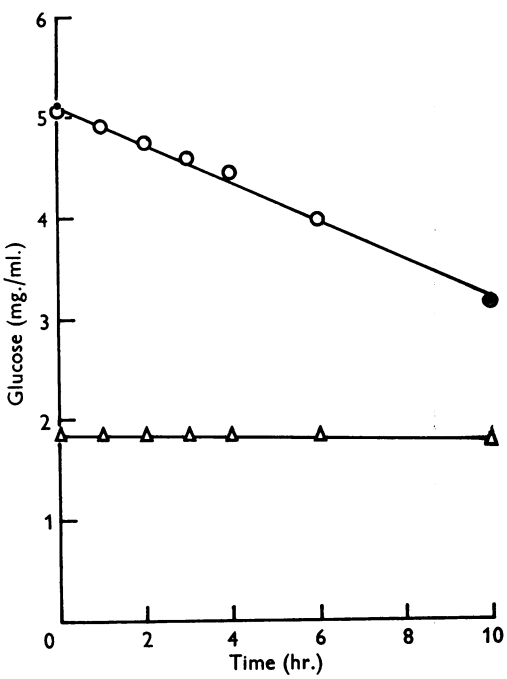

Fig. 3. Fermentation by *E. caudatum* in the presence of glucose. ○, Starch (as glucose) in protozoa incubated with glucose; ●, starch (as glucose) in protozoa incubated without glucose; △, glucose in solution. For conditions see text.

chromatographically detectable effect on lactose, raffinose, xylobiose and wheat-flour pentosan (Howard, 1957). Very slight hydrolysis of cellobiose, trehalose, melibiose, α-methyl glucoside, sucrose, inulin and bacterial levan was observed, but significant hydrolysis occurred of amylose, amylopectin, glycogen, β-dextrin and maltose only. Glucose and maltose were the main products from the polysaccharides, with smaller amounts of a substance chromatographically indistinguishable from maltotriose. With the decrease of the 'blue value' of amylose during incubation for 30 min. as a measure of activity, the amylase in the extracts was tested under different conditions. The variations of activity with pH and temperature are shown in Figs. 4 and 5 respectively. The optimum pH at 38° was 5·5, and the optimum temperature at pH 7 was 38°. The amylase appears to be unstable at 45°, since the activity at this temperature during incubation for 1 hr. was less in proportion to activity at the optimum temperature, than during 30 min. In 0·2 M-sodium citrate buffer, pH 7, the amylase activity was unchanged by the addition of NaCl to a final concentration of 0·14 M. In 0·2 M-sodium acetate buffer, pH 6·9, the amylase activity was decreased by about 23 and 40% when $CaCl_2$ was present in concentrations of 0·017 and 0·07 M respectively. When the cell

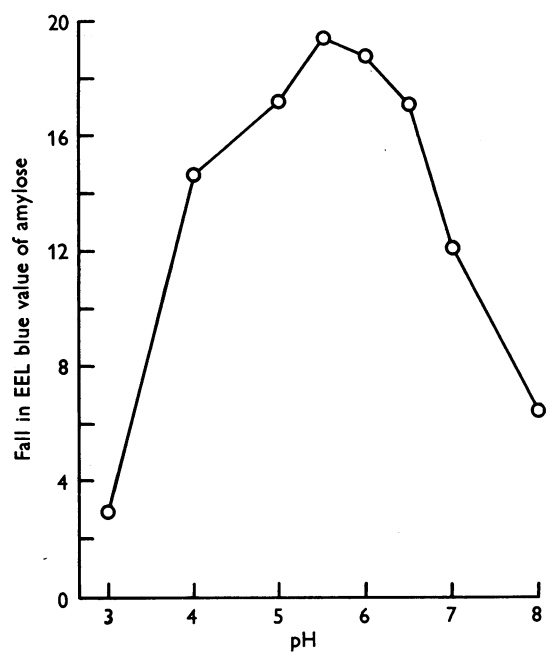

Fig. 4. Variation of activity of *E. caudatum* amylase with pH. Reactions were studied in 0·1 M-sodium citrate buffers of pH indicated. For other conditions see text.

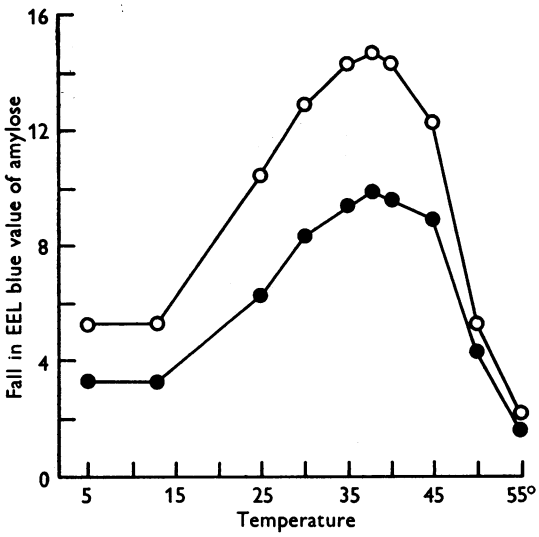

Fig. 5. Variation of activity of *E. caudatum* amylase with temperature. ●, Activity during incubation for 30 min.; ○, activity during incubation for 60 min. For conditions see text.

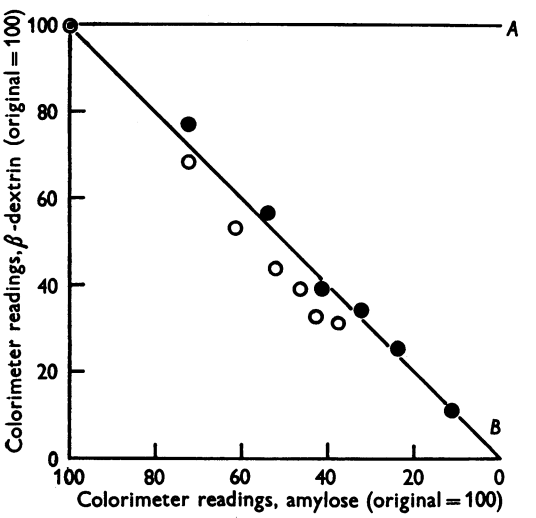

Fig. 6. Comparison of the action of salivary amylase and *E. caudatum* amylase on amylose and β-dextrin. *A*, Theoretical curve for β-amylase; *B*, theoretical curve for α-amylase. ●, *E. caudatum* amylase; ○, human salivary amylase. Amylose digests contained 1 mg. of amylose/ml. Samples (1 ml.) were diluted with iodine solution (I_2, 2 mg.; KI, 20 mg., in 100 ml. of water) and readings obtained in an EEL colorimeter (Evans Electroselenium Ltd.) with an Ilford no. 608 filter. β-Dextrin digests contained 1 mg. of β-dextrin/ml. Samples (2 ml.) were diluted with iodine solution (I_2, 4 mg.; KI, 40 mg., in 100 ml. of water) and readings obtained with an Ilford no. 626 filter. All digests were incubated at 38°.

Table 3. *Fractionation of amylase and maltase in a cell-free extract of* Entodinium *by the method of Hockenhull & Herbert* (1945)

Fraction	Activity (% of original) Amylase	Maltase
Crude extract	100	100
Ethanol solution after starch treatment	37	45
Phosphate extract	59	0
Recovery	96	45

extract was allowed to act upon amylose and β-dextrin simultaneously, and the effects on the two substrates were calculated and plotted as described by Hobson & MacPherson (1952), the graph shown in Fig. 6 was obtained. A similar experiment, in which centrifuged and diluted human saliva was used, gave similar results, which are also shown in Fig. 6.

The maltase in the protozoal extracts had maximum activity at pH 5 in the citrate buffer.

Treatment of a cell-free extract according to the method of Hockenhull & Herbert (1945) yielded two fractions, one an ethanolic solution containing material not adsorbed on to the potato starch, and another containing material originally adsorbed on to the starch, and extractable into phosphate buffer at pH 5·8. The amylase and maltase activities of these solutions, compared with the activities of the crude extract, are shown in Table 3. When the purified amylase acted upon amylose the main product was maltose; a little maltotriose and a trace of glucose were also formed.

DISCUSSION

The genus *Entodinium* Stein contains a large number of species, all of which have been found only in the rumen. In addition to the differentiation of these species on anatomical grounds, the entodinia can also be grouped physiologically by their feeding habits, according to the materials observed within the cells. Thus Bhatia (1936) mentions other ciliates, flagellates, bacteria, pollen grains, 'vegetable particles' and 'small plant débris' as forming the food of different *Entodinium* species. Clearly the genus is very heterogeneous biochemically, and little useful information would be obtained from suspensions of mixed *Entodinium* species. We have been fortunate in that the preparations we have used consisted very largely of *E. caudatum*. It has been known for some time from microscopic observations that this species is primarily a starch feeder (Hungate, 1943), whereas the minor constituents of the suspensions, those Protozoa resembling *E. simplex*, are most probably not. We consider that the properties of the sus-

pensions used in this work can be taken to be the properties of *E. caudatum* alone.

The present biochemical observations suggest that *E. caudatum* satisfies its carbohydrate requirements wholly by the ingestion and fermentation of starch grains, and not by any other means. Thus none of the soluble sugars tested, nor a solution of soluble starch, was fermented during incubation for 10 hr., nor did these substances affect the course of the endogenous fermentation of the starch stored within the cells.

The products formed during starch fermentation by *E. caudatum* resemble qualitatively those formed by numerous other carbohydrate-fermenting bacteria and Protozoa of the rumen, namely, volatile fatty acids, lactic acid, carbon dioxide and hydrogen. In making a quantitative determination of fermentation products, a difficulty was encountered which we were unable to overcome. To measure the production of carbon dioxide and volatile fatty acids by the same suspension of protozoa would require a buffer solution containing neither bicarbonate nor acetate. Such solutions, buffered only by phosphate, were invariably harmful to *Entodinium* suspensions, so that the protozoa were dying after incubation for 6 hr. Simultaneous measurement of carbon dioxide and volatile acid production has therefore proved impossible so far. The buffer solution containing acetate maintained the entodinia in a healthy state long enough for a short-term (6 hr.) experiment to be carried out, in which the fermentation gases were analysed. These gases were also analysed in 1 hr. experiments in the Warburg apparatus; these results suggest a relatively slightly lesser conversion of starch into gases during the earlier part of the fermentation period (Table 1). The lactic acid and volatile fatty acids were measured in other experiments, with the bicarbonate buffer; a longer incubation period (24 or 48 hr.) was required here, in order to obtain enough volatile acids for analysis. It is clear that a true carbon balance and hydrogen:oxygen ratio cannot be calculated with certainty from our results. If, however, it is assumed that the form of starch fermentation by *E. caudatum* is the same over different periods, and in different buffer solutions, apparent carbon balances and hydrogen:oxygen ratios may be calculated. When this is done, the carbon recovery appears to be 96 % of the carbon in the starch fermented, and the hydrogen:oxygen ratio in the products appears to be 2·1. Although a great deal of reliance cannot be placed in these two figures, we believe they show that no major product of starch fermentation has been overlooked in our analyses.

The most striking feature of the results is the low proportion of starch converted into lactic acid by *E. caudatum*, when compared with other ruminal starch-fermenting organisms. The distribution of carbon in the products is shown in Table 4, with the corresponding figures for endogenous fermentation of storage starch by rumen holotrichs for comparison. The products of fermentation of added glucose by the holotrichs may also justifiably be included here. An important starch-decomposing bacterium in the rumen, *Streptococcus bovis*, produces lactic acid and volatile acids, but no carbon dioxide. The proportions of lactic acid and volatile acids vary widely with conditions of culture (Dr P. N. Hobson, personal communication), so that it is not possible to include figures for this organism also in Table 4. The low rate of lactic acid production characteristic of *E. caudatum* could be of value to an animal whose rumen contains a high proportion of this organism. The sudden ingestion of large amounts of starch can be harmful to ruminants, because of the rapid production of large amounts of lactic acid, and the consequent fall of pH of the rumen contents to an abnormally low level (Scarisbrick, 1954). The presence of micro-organisms which can not only store away added starch for a more leisurely digestion, but can also, during the course of this digestion, produce insignificant amounts of lactic acid, would clearly be of benefit to the ruminant in preventing the development of this undesirable acidity.

A surprising result of our manometric experiments is that, although starved entodinia readily swallow rice-starch grains, no increase in fermentative activity is seen, at least during the first hour. Only after a longer period is an increase found (Fig. 2). This contrasts with the observation that *Isotricha* suspensions increase their rate of fermentation immediately on adding rice starch (Howard, 1959*a*).

Table 4. *Distribution of carbon in the products of fermentation of starch or glucose by rumen Protozoa*

Results are expressed as percentage of carbon in total carbon of the fermentation products.

Organism and substrate	Products		
	Lactic acid	Volatile fatty acids	CO_2
Isotricha + *Dasytricha* storage starch*	53	32	15
Isotricha + *Dasytricha* added glucose†	38	44	18
Dasytricha added glucose‡	48	31	22
Entodinium caudatum ingested starch§	2	80	19

* Calculated from table 4 of Heald & Oxford (1953).
† Calculated from table 2 of Heald & Oxford (1953).
‡ Calculated from table 1 of Gutierrez (1955).
§ Present results.

In the cell-free extracts made from the *Entodinium* suspensions, the only carbohydrase activities found in significant concentration were amylase and maltase. This finding agrees with the previous conclusion that *E. caudatum* relies solely on starch for satisfying its carbohydrate needs. The removal of all the maltase activity from the amylase, together with the chromatographic evidence and the effect of enzyme action on the iodine-staining properties of amylose, amylopectin and β-dextrin, show the enzyme to be a fairly typical α-amylase. The *E. caudatum* α-amylase is not identical with any of the α-amylases already investigated in rumen micro-organisms. Those of the holotrichs *Dasytricha* and *Isotricha* examined by Mould & Thomas (1958) have optimum pH values of 5·0 and 6·0 (*Dasytricha*) and 4·8 (*Isotricha*). The α-amylase of the rumen oligotrich *Epidinium caudatum* (Bailey, 1958) has maximum activity at pH 6, and an optimum temperature range of 37–45°. *S. bovis* α-amylase has maximum activity at pH 5·5–6·5, and an optimum temperature of 48° (Hobson & MacPherson, 1952). The distinction between this latter enzyme and the *Entodinium* amylase is particularly important. Gutierrez & Davis (1959) have demonstrated the ingestion by species of *Entodinium*, including *E. caudatum*, of large numbers of bacteria, identified by subsequent culture as *S. bovis*. The differences between the amylase in the *Entodinium* preparations and that of *S. bovis* show that the enzyme is a truly protozoal product, not a bacterial one.

SUMMARY

1. Active suspensions, consisting almost wholly of *Entodinium caudatum*, and free from bacteria, have been obtained from the rumen liquor of a sheep.

2. The protozoal cells in freshly prepared suspensions were filled with grains of plant starch, which gradually disappeared during incubation. The principal products formed during the fermentation were volatile fatty acids (chiefly butyric acid and acetic acid, with a little propionic acid and formic acid), carbon dioxide, hydrogen and a little lactic acid.

3. The *E. caudatum* suspensions did not metabolize sugars, or soluble starch, given in solution.

4. *E. caudatum* readily swallowed grains of rice starch, but this did not cause an immediate rise in the fermentation rate.

5. Cell-free extracts were prepared by grinding the suspensions with carborundum. Strong maltase and amylase activity was found in the extracts, but only traces of other carbohydrases. The amylase was examined in detail, and compared with similar enzymes in other rumen protozoa. It is an α-amylase.

6. It is concluded that *E. caudatum* satisfies its carbohydrate requirements solely by the ingestion of granular starch.

We are greatly indebted to our colleague, Dr J. M. Eadie, for placing the sheep at our disposal, and for her advice on the Protozoa; also to our colleague, Dr P. N. Hobson, for the gift of starch materials and for advice on a number of aspects of this work. We thank Mrs M. G. Campbell, Miss C. M. Davidson and Mr W. Shand for their assistance.

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