The Biochemistry of Rumen Protozoa

7. THE CARBOHYDRASES OF *POLYPLASTRON MULTIVESICULATUM* (DOGIEL & FEDOROWA)*

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(Received 7 March 1963)

Polyplastron multivesiculatum (Dogiel & Fedorowa) is one of the larger Ophryoscolecoid ciliates which occurs frequently in rumen protozoal populations, particularly in sheep (Eadie, 1962*b*). This organism has five skeletal plates, but the smallest of these is difficult to distinguish except in very empty organisms. Because of this, as explained by Eadie (1962*b*), there has been much confusion in the identification of *P. multivesiculatum*, e.g. when Sugden (1953) examined this species but considered it to be '*Metadinium medium* Awerinzew & Mutafova'. Sugden (1953) reported that, *in vitro*, washed suspensions of this organism rapidly ingested rice starch grains and cotton fibres, and that the latter prolonged the life of the organisms and appeared to disintegrate within the cell. Hungate (1943) had previously observed the ingestion of cellulose by this organism (under the name *Diplo-dinium multivesiculatum*) when grown in culture. The experiments of Sugden (1953) were made possible by the fortuitous occurrence in one sheep for a short time of '*M. medium*' as the predominant ciliate. A more prolonged investigation of the organism has been made possible by the establishment of it as the sole large ciliate in an isolated sheep (Eadie, 1962*a*). The present paper reports the investigation into the carbohydrases present in extracts of *P. multivesiculatum*.

MATERIALS AND METHODS

Sheep as source of protozoa. A rumen-cannulated Grey-face wether, maintained on a ration of 300 g. of hay and 150 g. of concentrates, fed twice daily, was used throughout this work. It had been kept free from ciliates from birth, and after cannulation it was placed in an isolation crate (Eadie, 1962*a*). Before the present series of experiments small entodinia had been established in the rumen. A suspension of *P. multivesiculatum* (about 15 individuals), which had been separated from a mixed population under a dissection microscope, was then inoculated directly into the rumen. The population increased to a suitable level within 3 weeks.

Preparation of extracts of protozoa. Samples (250 ml.) of

rumen liquor were withdrawn 1.5 hr. after the morning meal, and treated as described by Abou Akkada & Howard (1960), except that the addition of mannose to kill holotrich protozoa was unnecessary. The small entodinia were removed from the suspension by washing by decantation, and a clean motile preparation of *P. multivesiculatum* was obtained 1.5 hr. after removing the rumen liquor from the sheep. The protozoa were stored at -20° until required. Cell-free extracts were obtained from the protozoa by the procedure of Abou Akkada & Howard (1960), except that the cells were broken by shaking the thawed suspension at room temperature in a disintegrator (Mickle, 1948) with an equal volume of glass Ballotini and citrate-phosphate buffer, pH 6.0 (10 ml.). The cells were all broken after shaking for 45 min.

Materials. These were the same as used by Abou Akkada & Howard (1960, 1961), with the following additions: standard-grade Whatman ashless cellulose powder; powdered cotton-wool prepared as described by Sugden (1953).

Buffer solutions. The following were used: (a) sodium phosphate-citric acid (McIlvaine, 1921); (b) 0.2M-sodium acetate (Gomori, 1955); (c) veronal (Michaelis, 1930).

Enzyme experiments. Qualitative tests for the hydrolysis of carbohydrates and quantitative experiments with amylase, polygalacturonase, pectin esterase and pentosanase were carried out as previously described (Abou Akkada & Howard, 1960, 1961; Howard, Jones & Purdom, 1960). The hydrolysis of cellobiose, lactose, maltose, melibiose and sucrose was measured by the glucose-oxidase method (Bailey & Howard, 1963*a*).

Powdered cellulose or cotton-wool (2.5 mg.), protozoal extract (0.25 mg.), phosphate-citrate buffer, pH 6.0 (0.2 ml.), and toluene (1 drop) were incubated in stoppered tubes for 48 hr. Cellulose and enzyme-control tubes were incubated at the same time. The reaction was stopped by immersing the tubes in boiling water for 5 min. Water (2 ml.) was added to each tube, and the insoluble material was collected by centrifuging. The residue was washed once with N-NaOH (1 ml.), twice with water (1 ml.) and then dissolved in 72% (w/v) H_2SO_4 (2 ml.) during 1 hr. at room temperature. The carbohydrate in this solution, and in the aqueous supernatant obtained in the first centrifuging, was determined by the anthrone method (Trevelyan & Harrison, 1952).

Other methods. The methods of paper chromatography and N analysis used in this work were those described by Abou Akkada & Howard (1961) and Howard (1959*a, b*). The incubation of enzyme digests was at 38° throughout this work.

* Part 6: Bailey & Howard (1963*a*).

RESULTS

Action of extracts of protozoa on carbohydrates

Starch fractions. The extracts of *P. multivesiculatum* readily hydrolysed amylose, amylopectin, β -dextrin and glycogen. Maltose, glucose and a smaller amount of maltotriose were the principal products from all these substrates, as judged from paper chromatograms. Quantitative experiments, in which the loss of iodine-staining power of amylose was used as a measure of activity, showed that the amylase was most active at pH 5.0 (Fig. 1). Under the conditions described by Abou Akkada & Howard (1960) the activity of the amylase was not altered by 0.14M-sodium chloride, and was decreased by 8 and 24 % in the presence of 0.014M- and 0.07M-calcium chloride respectively. A comparison of the action of *P. multivesiculatum* amylase and human salivary amylase on amylose and β -dextrin was made (Abou Akkada & Howard, 1960). The results are illustrated in Fig. 2. A portion of the crude protozoal extract was treated with starch according to the method of Hockenhull & Herbert (1945) for separating amylase and maltase. A preparation was obtained which possessed no maltase activity, but retained 70 % of the amylase activity of the original material. This purified amylase hydrolysed amylose, amylopectin

and β -dextrin. Maltose was the principal product; maltotriose and glucose were produced in small amounts. The response of the purified enzyme to the presence of Ca^{2+} and Cl^{-} ions was identical with that of the crude enzyme.

Cellulose. The degree of cellulolysis in these experiments was determined by measuring both the amount of carbohydrate in solution and the loss of insoluble carbohydrate at the end of the incubation period. Formation of soluble carbohydrate in the control tubes was negligible. The results of the two methods were in agreement and showed that 25 % of the cellulose powder or 30 % of the cotton wool was made soluble during incubation with the enzyme for 48 hr. The carbohydrate in solution was shown by paper chromatography to be mainly glucose, with a little cellobiose and a trace of xylose. Rumen liquor (60 ml.) collected from an isolated sheep whose rumen was free from ciliate protozoa (Eadie, 1962*a*), was centrifuged at 20000*g* for 30 min., and the sedimented bacteria were washed and submitted to the same process of disintegration, centrifuging and dialysis as was used in preparing the protozoal extracts. The bacterial extract caused 0.2 % conversion of substrate into soluble carbohydrate when incubated with cellulose under the same conditions as used with the protozoal extract.

Pentosan. Wheat-flour pentosan was readily hydrolysed by the protozoal extract. The products were mainly xylose and arabinose, with small amounts of xylobiose and other oligosaccharides. In the quantitative experiments, in phosphate-

Fig. 1. Variation of activity of some carbohydrases of *Polyplastron multivesiculatum* with pH. ●, Amylase; ○, pentosanase; △, invertase. The conditions of assay are described in the text.

Fig. 2. Comparison of the action of salivary amylase and *P. multivesiculatum* amylase on amylose and β -dextrin. A, Theoretical curve for β -amylase; B, theoretical curve for α -amylase. ●, *P. multivesiculatum* amylase; ○, human salivary amylase. The experimental conditions were as described by Abou Akkada & Howard (1960).

citrate buffer, the extract was most active at pH 6.3 (Fig. 1).

Pectic substances. Considerable amounts of reducing sugar were produced when the protozoal extract was incubated with pectin or sodium polypectate. This was shown by paper chromatography to consist almost wholly of galacturonic acid. The hydrolysis of sodium polypectate, expressed as mg. of reducing sugar as galacturonic acid produced/hr./mg. of protein N was 15.7 with one batch of enzyme at pH 7.9, and 11.7 with another batch at pH 6.0. Expressed in the same terms, the hydrolysis of pectin under the conditions of the latter experiment was 5.4. In 0.2M-sodium acetate buffer, pH 6.0, the polygalacturonase activity of the extract remained unchanged on the addition of calcium chloride up to a concentration of 0.1 mM; higher concentrations of calcium chloride depressed activity by precipitating the substrate.

The protozoal extract liberated methanol from pectin, in addition to causing glycosidic hydrolysis. The pectin esterase was most active at pH 7.2, and at this pH 4.4 mg. of methanol was produced/hr./mg. of protein N in the extract.

Oligosaccharides and other carbohydrates. Methyl α -D-glucoside, $\alpha\alpha$ -trehalose and bacterial levan were not hydrolysed by the protozoal extract. Sucrose, maltose, cellobiose, lactose, melibiose, raffinose, salicin, arbutin, aesculin, inulin and xylobiose were all hydrolysed to an appreciable extent. The raffinose hydrolysate contained fructose, with smaller amounts of glucose and galactose, and a little melibiose, but no sucrose. The inulin hydrolysate contained only fructose. In phosphate-citrate buffer, pH 6.0, the hydrolysis of some of the disaccharides, expressed as μ moles of substrate hydrolysed/hr./mg. of protein N in two batches of extract respectively was: sucrose, 77, 27; cellobiose, 49, 14; maltose, 2, 1; lactose, 11, 7; melibiose, 16, 8. The hydrolysis of sucrose was greatest at pH 5.6 (Fig. 1).

DISCUSSION

If it be accepted that the presence of carbohydrases in cell-free extracts of rumen protozoa to some extent reflects the feeding habits of the living organism, the finding of amylase and maltase in the extracts of *Polyplastron multivesiculatum* is to be expected, since Sugden (1953) observed that rice and potato starch grains were utilized by this organism in washed suspensions. All our observations on the amylase of *P. multivesiculatum* show that, like the amylases of all other rumen bacteria and protozoa which have been examined, it is of the α -type. It resembles the amylase of *Entodinium caudatum* in being unaffected by Cl^- ions and inhibited by Ca^{2+} ions, and in yielding maltose

as the main product of starch hydrolysis (Abou Akkada & Howard, 1960). Its pH optimum, pH 5.0, is a little lower than those of some other rumen Ophryoscolecids [*Epidinium ecaudatum*, pH 6.0 (Bailey, 1958); *Entodinium caudatum*, pH 5.5].

Hungate (1943) was able to grow *P. multivesiculatum* in culture *in vitro* together with *Entodinium caudatum* for 100 days when grass, cellulose (acid-treated cotton) and ground wheat were supplied as food. When the *P. multivesiculatum* were separated from these cultures and were well washed and ground with sand, extracts were obtained that produced reducing sugars when incubated with cellulose. The debris in the cultures, which contained most of the bacteria and cellulose, yielded extracts which produced no reducing sugars when incubated with cellulose. Hungate (1943) therefore concluded that the protozoa were themselves able to digest the cellulose.

Sugden (1953) found that cellulose was particularly effective in prolonging the life of *P. multivesiculatum* in cultures, and that granules of storage starch were deposited in the skeletal plates and around the periphery of the cell when cellulose fibres were seen to be disintegrating within the organisms. However, when bactericidal concentrations of streptomycin were included in the medium, Sugden (1953) found that this conversion of cellulose into starch was diminished and the organisms died within a few days. For this reason she considered it probable that the protozoa were unable to make use of cellulose without the aid of symbiotic bacteria.

Halliwell (1957) criticized the conclusion of Hungate (1943) on the ground that no quantitative evidence of cellulose decomposition was obtained, and considered that bacteria were responsible for the apparent decomposition of cellulose by rumen protozoa.

On the other hand B. Sugden & A. E. Oxford (personal communication) found that, in the presence of 0.8 mM-streptomycin, a suspension of *P. multivesiculatum* (initial count 9.7×10^5 ; final count 3.1×10^5 organisms) digested in 4 days 200 mg. of the 300 mg. of cellulose originally supplied.

In our experiments cellulase activity was assayed both by the loss of insoluble material and by the liberation of carbohydrate into solution when either chromatography-grade cellulose powder or acid-treated cotton-wool was the substrate. Appreciable activity against both substrates was found in the cell-free extracts of *P. multivesiculatum*. The method of extraction was ineffective in releasing any cellulase from a suspension of mixed rumen bacteria very many times greater in numbers than could occur in the protozoal suspension. Since these bacteria came from a sheep

without rumen ciliate protozoa, they must have been responsible for the whole of the cellulolytic activity in the rumen-liquor sample taken. The cellulase present in our extracts must therefore come solely from the protozoa. This confirms the conclusion of Hungate (1943) that *P. multivesiculatum* does itself produce cellulase and may therefore be justifiably regarded as a true symbiont within the ruminant.

The pectic enzymes of *P. multivesiculatum* may be compared with those of other rumen protozoa: *Isotricha* (Abou Akkada & Howard, 1961) and the mixture of *Isotricha*, *Dasytricha* and *Epidinium ecaudatum* investigated by Wright (1960). When the polygalacturonase of *P. multivesiculatum* acts on polygalacturonic acid, galacturonic acid is the principal product; the enzyme of *Isotricha* yields little galacturonic acid, but much oligouronide material. The *P. multivesiculatum* enzyme in this respect resembles more closely that in the extract of mixed protozoa used by Wright (1960). The pectin esterase of *P. multivesiculatum* has a much lower pH optimum (pH 7.2) than that of *Isotricha* (pH 8.6–8.8).

An enzyme system hydrolysing xylan and arabino-xylans was first found in rumen protozoa by Bailey, Clarke & Wright (1962), studying *Epidinium ecaudatum*. Arabinose and xylobiose were the principal products of hydrolysis. The enzyme complex of *P. multivesiculatum* seems, as far as we have examined it, to be broadly similar to that of *Epidinium ecaudatum*.

Several oligosaccharides were hydrolysed by the *P. multivesiculatum* extracts. The presence of maltase, cellobiase and xylobiase in the extracts is clearly connected with the presence of amylase, cellulase and xylanase. In the living organism the former group of enzymes is probably necessary to render the initial hydrolysis products of the polysaccharides available for further metabolism. The production of fructose from sucrose and raffinose, and possibly also its production from inulin, is evidence of a moderate invertase activity in the extracts. The holotrich rumen protozoa are extremely active in invertase production (Howard, 1959*b*; Thomas, 1960), and the occasional finding of traces of this enzyme in preparations from other rumen protozoa has usually been ascribed to slight contamination of the suspensions of the organisms with a few holotrich individuals (Bailey, 1958; Bailey & Howard, 1963*a*). Such contamination is impossible in the present work, since there are no holotrichs in the rumen from which the *P. multivesiculatum* suspensions are prepared. The invertase in the extracts we therefore consider to be a true product of this protozoon. The hydrolysis of melibiose and lactose by the extracts may reflect the ability of the organism to utilize the α - and β -linked

galactose found in many plant lipids (Weenink, 1961; Bailey & Howard, 1963*b*).

This survey of the carbohydrases in extracts of *P. multivesiculatum* suggests that the organism is equipped to decompose all the major polysaccharides (cellulose, hemicellulose, starch, pectin and fructan) that occur in the food of ruminants, as well as some of the soluble sugars that are also present. In this respect it seems to be the most versatile of all the rumen protozoa so far studied.

SUMMARY

1. *Polyplastron multivesiculatum* has been established as the sole large protozoon in the rumen of a sheep. Suspensions of the organism, substantially free from bacteria and other ciliates, have been obtained from the rumen liquor of this sheep.

2. Cell-free extracts obtained from disintegrated cells of *P. multivesiculatum* hydrolyse a number of poly- and oligo-saccharides.

3. A high α -amylase activity is present in the extracts. This enzyme has maximum activity at pH 5.0, and yields maltose as the main product of hydrolysis of amylose. The extracts also hydrolyse maltose, but not methyl α -D-glucoside or $\alpha\alpha'$ -trehalose.

4. The extracts slowly hydrolyse cellulose, yielding glucose as the main product. Cellobiose is also hydrolysed. Extracts of mixed rumen bacteria, prepared in the same way as the protozoal extracts, do not hydrolyse cellulose.

5. Wheat-flour pentosan and xylobiose are hydrolysed by the extracts. The former is most readily decomposed at pH 6.3, and the main products are arabinose and xylose.

6. The extracts hydrolyse pectin and sodium polypectate. Galacturonic acid is the main product from both substrates. Pectin-esterase activity is greatest at pH 7.2.

7. Sucrose, melibiose, raffinose, inulin and lactose are also hydrolysed by the extracts.

A. R. Abou Akkada gratefully acknowledges the receipt of a grant from The Wellcome Trust during this work. We thank Dr P. N. Hobson of the Rowett Research Institute and Dr K. el-Shazly of the Faculty of Agriculture, University of Alexandria, for their advice and encouragement. Mr W. Shand and Mr R. Summers gave valued assistance in our experiments.

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Biochem. J. (1963) **89**, 272

Separation and Characterization of Two Rat-Intestinal Amylases

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(Received 16 April 1963)

During a recent study of the carbohydrases of rat-intestinal mucosa, the amylase activity was found to yield two separate peaks on ion-exchange chromatography (Dahlqvist, 1963). This paper deals with the characterization of the two enzymes responsible for the amylase activity of these extracts.

MATERIALS AND METHODS

Determination of carbohydrase activities. The amylase activity was measured by the method described by Dahlqvist (1961*a*). One unit of amylase activity is now defined as the activity releasing reducing groups corresponding to 1 μ mole of maltose/min. at 37° (Dahlqvist, 1963). The disaccharidase activities were assayed with a tris-buffered glucose-oxidase reagent (Dahlqvist, 1961*b*), under the same incubation conditions as those described for the pig enzymes (Dahlqvist, 1960, 1961*b*). One unit of disaccharidase activity is defined as the activity hydrolysing 1 μ mole of disaccharide/min. at 37°.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with the modified 'reagent B' introduced by Eggstein & Kreutz (1955). A standard curve was prepared with freshly dissolved human serum albumin, kindly supplied by AB Kabi (Stockholm, Sweden).

Thin-layer chromatography. The method of Weill & Hanke (1962), using a solvent mixture containing butan-1-ol-2,6-lutidine-water (6:3:1, by vol.), was used. Glucose and maltose (5 μ g. each) were used as markers, and 10 μ l. of the reaction mixture for amylase determination was applied to the plate after the reaction had proceeded for the desired time at 37° and had been interrupted by placing the tube in boiling water for 2 min.

Viscosimetry. The soluble starch preparation used for the reduction method of assay of amylase (Dahlqvist, 1961*a*) yields a solution with very low viscosity and is therefore not suitable for viscosimetric studies. For this purpose a solution prepared from commercial rice starch was used instead. The substrate solution was prepared by suspending 2.0 g. of rice starch in 100 ml. of 0.1M-phosphate-buffered NaCl at pH 6.9 (3.03 g. of KH_2PO_4 , 3.96 g. of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 0.400 g. of NaCl diluted with water to 1000 ml.). The suspension was placed in a boiling-water bath for 15 min. with automatic stirring, and left to cool. A suitably diluted enzyme solution was incubated at 37° with an equal volume of the buffered substrate and the reaction stopped by mixing 5 ml. of the reaction mixture with 0.5 ml. of 1N-acetic acid. The viscosity was determined in an Ostwald viscosimeter and the relative viscosity (viscosity/viscosity of water) calculated. With the same substrate, the liberation of reducing groups was determined with the 3,5-dinitrosalicylate reagent of Sumner (1924), as in the usual assay procedure for amylase (Dahlqvist, 1961*a*). The reaction was stopped by heating the tubes in boiling water for 2 min.

Animals. Sprague-Dawley rats of both sexes, weighing 150–200 g., were used.

Mucosal homogenates. The rats were starved for 12–18 hr., with free access to water, and then killed by a blow on the head. The proximal two-thirds of the small intestine was removed and immediately placed in ice-cold 0.9% NaCl. The intestine was then cut open, rinsed with chilled 0.9% NaCl, blotted gently and the mucosa scraped off with a glass slide. After weighing, the mucosa was mixed (1 g./4 ml.) with ice-cold 0.2M-sodium phosphate or 0.1M-sodium maleate buffer, pH 7.0. Several drops of octan-2-ol were added to prevent foaming and the mixture was homogenized for 2 min. with an Ultra-Turrax homogenizer.