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# The Biochemistry of Rumen Protozoa

## 6. THE MALTASES OF *DASYTRICHA RUMINANTIUM*, *EPIDINIUM ECAUDATUM* (CRAWLEY) AND *ENTODINIUM CAUDATUM**

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The digestion of starch is an important feature of the rumen fermentation, and a number of micro-organisms which decompose starch have been found in rumen contents. Several types of protozoa are active in this decomposition, and the amylases of the following species have been examined in detail: mixed *Isotricha intestinalis* plus *I. prostoma*, and *Dasytricha ruminantium* (Mould & Thomas, 1958), *Epidinium ecaudatum* (Crawley) (Bailey, 1958) and *Entodinium caudatum* (Abou Akkada & Howard, 1960). The amylases of these organisms are all of the α-type, and yield maltose as the main product of amylose hydrolysis. With the exception of the *Isotricha* species, these protozoa also produce maltase; some of the properties of this enzyme in the three species concerned are described here.

## MATERIALS AND METHODS

*Enzymes.* The dialysed and freeze-dried extracts of *Dasytricha ruminantium* and *Entodinium caudatum* were prepared by the methods of Howard (1959) and Abou Akkada & Howard (1960) respectively. The freeze-dried powders each contained about 9% of N. The extracts of *D. ruminantium* contained amylase, invertase, cellobiase, β-glucosidase and pectinase in addition to maltase (Mould & Thomas, 1958; Howard, 1959; Abou Akkada & Howard, 1961). The extracts of entodinia contained amylase, proteinase and peptidase in addition to maltase (Abou Akkada & Howard, 1960, 1962).

Epidinia were disrupted, and water and phosphate buffer extracts made, as described by Bailey, Clarke & Wright (1962). The extracts were freeze-dried. Three such extracts, I, II and III, were used, obtained from three batches of protozoa. All corresponded to 'solution D' (phosphate buffer extract) of Bailey *et al.* (1962), except that in the preparation of I and II the second water-extraction step ('solution B') was omitted. The three freeze-dried powders contained respectively 1·1, 0·5 and 0·6% of N. The presence in these materials of carbohydrase activities other than maltase was investigated. Enzyme powder (5 mg.) and carbohydrate (2–5 mg.) were incubated for 24 hr. in citrate buffer, pH 6·0 (0·4 ml.). The solutions were examined by paper chromatography before and after incubation.

*Carbohydrates.* These were from the usual commercial sources except for the following compounds. Commercial maltose was freed from traces of glucose and maltotriose by fractionation on charcoal–Celite columns. Maltodextrins were isolated from a partial acid hydrolysate of amylose (Whelan, Bailey & Roberts, 1953). Maltitol and malto-

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tri-itol were prepared from the corresponding sugars by reduction with sodium borohydride (tetrahydroborate). Wheat-flour arabinoxylan was prepared by the method of Howard (1957), isomaltose from a partial acid hydrolysate of dextran (Bailey & Roberton, 1962), and amylose from potatoes (Hobson, Pirt & Whelan, 1951).

*Chromatographic analyses.* Solutions were desalted with Bio-Deminrolit ($CO_2$ form) (The Permutit Co. Ltd.), and analysed by paper chromatography, with the solvents and spray reagents described by Bailey & Roberton (1962). Oligosaccharides were isolated or purified by fractionation on charcoal–Celite columns, with aqueous ethanol containing formic acid as eluent (Taylor & Whelan, 1962). The dried fractions were treated with anhydrous methanol to remove inorganic material, freeze-dried and finally dried *in vacuo* at 60°.

*Buffer solutions.* The buffers used were: (*a*) 0·2 M-sodium phosphate ($Na_2HPO_4$–$NaH_2PO_4$); (*b*) 0·1 M-sodium citrate; (*c*) 0·2 M-$Na_2HPO_4$–0·1 M-citric acid (McIlvaine, 1921).

*Maltase assay.* The glucose liberated by the action of the enzyme on maltose was determined by a colorimetric glucose-oxidase method. 25 mM-Maltose (0·1 ml.), buffer solution (0·3 ml.) and enzyme solution (0·1 ml.), in stoppered tubes, were placed in a water bath, together with appropriate controls and glucose standards. The concentration of maltose in the solution was thus 5 mM, or 0·18%. Except where stated otherwise, incubation was at 38°. At the end of the incubation period, which was usually 2 hr., the enzyme action was stopped by immersing the tubes for 5 min. in boiling water. The glucose content of the solutions was then determined by the method of Huggett & Nixon (1957), with the following modifications: (i) a purer preparation of glucose oxidase was used (Type II, Sigma Chemical Co., St Louis, Mo., U.S.A.); (ii) the reagent was made up in the tris buffer of Dahlqvist (1961) to suppress the maltase activity of the glucose-oxidase preparation. In experiments in which the liberation of glucose from substrates other than maltose was to be measured, the concentration in the incubation mixture was 2 mM.

*Transferase experiments.* An appropriate weight of enzyme (*D. ruminantium*, 1 mg.; entodinia, 0·5 mg.; epidinia preparations II or III, 5 mg.) was incubated with maltose for 24 hr. in citrate buffer, pH 6·0 (0·4 ml.). The solutions were then examined for oligosaccharides by paper chromatography.

## RESULTS

Hydrolysis of maltose by extracts of the three protozoal species was linear with respect to time and enzyme concentration when less than 5% of substrate had been decomposed. Negligible amounts of glucose were formed when the preparations were incubated alone, and none of them caused any loss of glucose when incubated with it. At 38° and optimum pH, hydrolysis of maltose, expressed as μmoles decomposed/hr./mg. of N, was: for *D. ruminantium*, 10; for *Entodinium caudatum*, 20; and for preparations I, II and III of *Epidinium ecaudatum*, 7, 11 and 11 respectively. These epidinia preparations possessed weak activity against five of the carbohydrates normally hydrolysed by the water extract (Bailey *et al.* 1962), namely amylose, arabinoxylan, melibiose, isomaltose and sucrose, but none of them hydrolysed αα-trehalose, methyl α-D-glucoside, turanose, or a wide range of substances containing β-linked glucose. The epidinia preparations produced no chromatographically detectable sugars when incubated alone.

The amylase activity of epidinia preparations II and III was measured by following the loss in iodine-staining power of a standard amylose solution. The composition of the solution and the staining procedure were as used by Bailey (1958), except that solid enzyme (5 mg.) was used, and the volume was adjusted with additional buffer solution. A 50% loss of iodine colour was obtained in 3–4 hr. with preparation II (25 μg. of N) and in 10–11 hr. with III (30 mg. of N). In contrast, a freeze-dried aqueous extract of epidinia (1 mg., containing 94 μg. of N) caused a 50% loss of iodine-staining power in 15–20 min.

*Effects of* pH *and of buffer.* In some cases maltase activity was affected by the buffer salts used (Fig. 1). The maltase of the epidinia (preparation I) was, over the pH range 5·5–7·0, only two-thirds to three-quarters as active in phosphate buffer as in citrate or citrate–phosphate buffers. Maximum activity occurred at pH 6·5 in phosphate, and at 6·7 in citrate–phosphate buffer. The maltase of entodinia also, when incubated in phosphate, had only three-quarters of the activity that it had in citrate buffer. This enzyme had maximum activity at pH 5·7–6·1 in both the citrate and the phosphate buffers. *D. ruminantium* maltase was equally active in citrate, phosphate or citrate–phosphate buffers over the pH range 5·0–6·0, and maximum activity was at pH 5·5 in each case.

*Effects of temperature.* Preparations of the enzymes of the three protozoal species were incubated with maltose under standard conditions, but at different temperatures. Citrate–phosphate buffer was used, and the results shown in Fig. 2 were obtained when each enzyme was tested at the pH found to give maximum activity at 38°. The maltases of entodinia and epidinia (preparation I) had optima at 38°, that of *D. ruminantium* at 50°. Similar results were obtained when all three enzymes were tested at the same pH, namely 6·0.

*Hydrolysis of other substrates.* The amounts of glucose liberated from maltotriose by the enzymes from *D. ruminantium*, entodinia and epidinia (preparation III) were respectively 44, 34 and 33% of the amounts liberated from maltose by the respective enzymes under the same conditions. The sugars in a duplicate digest of maltotriose and the epidinia enzyme were separated by paper chromatography, and then determined colorimetrically (Wilson, 1959) after spraying with aniline–

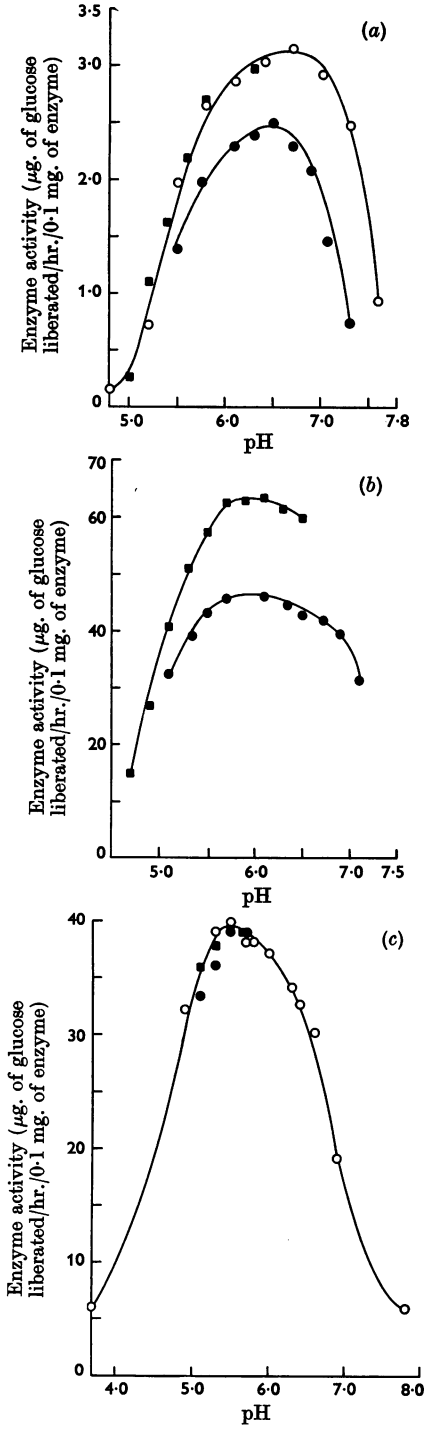


Fig. 1. Variation of activity of maltases of rumen protozoa with pH. The conditions are described in the text. (*a*) *Epidinium ecaudatum*; (*b*) *Entodinium caudatum*; (*c*) *Dasytricha ruminantium*. Buffer solutions: ■, citrate; ●, phosphate; ○, citrate–phosphate.

phosphate reagent (Howard, 1957), giving a maltose:glucose ratio of 1:2·0 by weight.

The epidinia preparation III liberated glucose from maltotetraose, maltopentaose and maltohexaose, also much more slowly than from maltose. In additional experiments solutions containing enzyme (3 mg.) and maltopentaose or maltohexaose (1·8 mg.), dissolved in citrate–phosphate buffer, pH 6·0 (1 ml.), were incubated for 12 hr. Portions (50 μl.) were removed at intervals, desalted and examined chromatographically. The substrate disappeared in 2–3 hr., and there was a concurrent early liberation of maltose and maltotriose from the pentasaccharide, and of maltose, maltotriose and maltotetraose from the hexasaccharide.

Glucose liberated from maltitol by the enzymes of all three protozoal species was less than 1 % of that liberated from maltose under the same conditions. Negligible hydrolysis of maltotri-itol occurred with the epidinia and entodinia enzymes, but the *D. ruminantium* preparation liberated glucose from this substance at 25 % of the rate for maltose. Paper chromatography showed unchanged maltotri-itol, glucose and maltitol; no maltose or sorbitol was present.

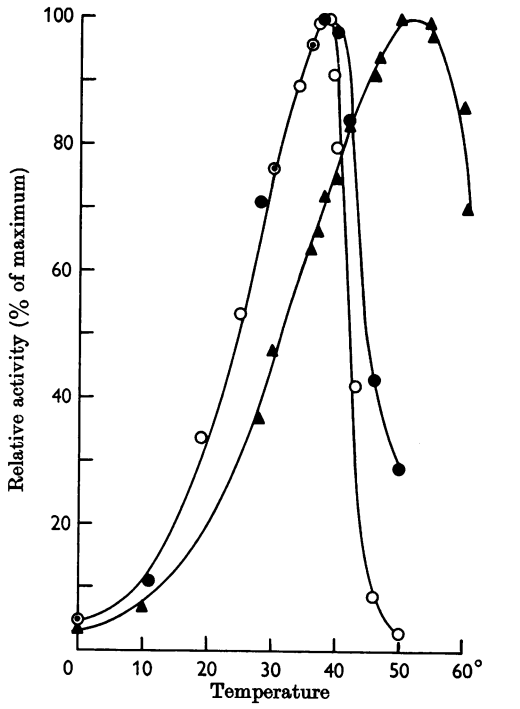


Fig. 2. Variation of activity of maltases of rumen protozoa with temperature. ○, *Epidinium ecaudatum* at pH 6·7; ●, *Entodinium caudatum* at pH 6·0; ▲, *Dasytricha ruminantium* at pH 5·5. Other conditions are described in the text.

*Transferase action of maltases.* The epidinia preparation II was tested against a range of concentrations (0·25–2·5 %, w/v) of maltose. Strong glucose spots were present on chromatograms of all the solutions. In addition, tri- and tetra-saccharide spots were present on the chromatograms of the solutions containing 1·25 and 2·5 % of maltose, but were barely detectable or absent from those containing 0·5 % or less. No spot corresponding to isomaltose was seen on any of the chromatograms. In several solvents, and as its *N*-benzylglycosylamine, the trisaccharide was chromatographically indistinguishable from authentic maltotriose. With the diphenylamine–aniline spray reagent it gave the typical blue colour which is specific for oligosaccharides containing a glycosidic link on C-4 of a reducing hexopyranose unit. The tetrasaccharide was similarly chromatographically indistinguishable from authentic maltotetraose. When the epidinia preparation was incubated in a solution containing 15 % (w/v) of maltose, the same tri- and tetra-saccharide spots appeared on chromatograms. No sign of panose or of any other trisaccharide was detected, although in the solvent systems used panose could be clearly separated from maltotriose and maltotetraose.

In a larger-scale experiment, epidinia preparation III (130 mg.) and maltose (270 mg.) were incubated in citrate buffer, pH 6·0 (11 ml.), for 24 hr. Fractionation of the sugars on charcoal–Celite yielded chromatographically pure trisaccharide (25 mg.) and tetrasaccharide (15 mg.). The purified trisaccharide had $[\alpha]_D^{18°} + 156°$ (*c* 0·18 in water); Whelan *et al.* (1953) reported $[\alpha]_D + 160°$ for maltotriose. Total acid hydrolysis (N-sulphuric acid for 2 hr. at 100°) of the trisaccharide yielded only glucose, and partial hydrolysis (0·3 N-sulphuric acid for 1 hr. at 100°) yielded substances chromatographically indistinguishable from glucose, maltose and unchanged starting material. No isomaltose was detected in the partial acid hydrolysate. The trisaccharide (10 mg.) was reduced with sodium borohydride and, after removal of boric acid, the product was subjected to partial acid hydrolysis. In addition to glucose, the hydrolysate contained a reducing sugar which was chromatographically indistinguishable from maltose, both in its mobility and in its colour reaction with the diphenylamine–aniline spray reagent.

The *D. ruminantium* and entodinia enzymes were tested for transferase activity at a maltose concentration of 2·5 % (w/v) only. The entodinia produced, in addition to much glucose, appreciable amounts of substances which, with all the chromatographic solvents and spray reagents used, were indistinguishable from maltotriose and maltotetraose. No isomaltose, panose or other trisaccharide was detected. The *D. ruminantium* enzyme produced from maltose two new substances, which were respectively indistinguishable from maltotriose and panose; as far as could be judged from the paper chromatograms, they were formed in approximately equal amounts. A considerable amount of glucose was present in the *D. ruminantium* digests at the end of incubation, but no isomaltose was produced.

To investigate any transferring activity that might be associated with the feeble invertase present in the epidinia extracts, preparation II was incubated in solutions containing 3, 5 or 15 % (w/v) of sucrose. A faint trisaccharide spot was detected on chromatograms of the 3 and 5 % solutions. The mixture of sugars present in these solutions was subjected to mild acid hydrolysis (0·1 N-sulphuric acid for 10 min. at 100°). Chromatograms of the hydrolysates showed spots corresponding to glucose and fructose but no maltose or other disaccharide.

*Rumen bacteria.* A suspension of mixed rumen bacteria, freed from protozoa by centrifuging as described by Bailey & Howard (1962), was incubated with antibiotics, and extracted with water and the phosphate buffer according to the procedures used to obtain the enzyme preparations from epidinia. The extracts so obtained were tested for maltase by the glucose-oxidase assay method. All were completely inactive.

## DISCUSSION

That the enzymes dealt with in the present work are genuine products of the protozoa, and are not derived from the few bacteria which still contaminate even the best preparations of rumen protozoa, is demonstrated by the results described in the preceding paragraph. Numbers of bacteria several orders of magnitude greater than could occur in the washed protozoal suspensions, when treated by the procedures used for obtaining the protozoal extracts, gave solutions which contained no maltase activity.

The enzymes of the three protozoal species which hydrolyse maltose appear to be true maltases, and not general α-glucosidases. It is known that *D. ruminantium* extracts cannot hydrolyse αα-trehalose or turanose (Howard, 1959), that *Entodinium caudatum* extracts have little or no action on methyl α-D-glucoside, αα-trehalose or sucrose (Abou Akkada & Howard, 1960), and that *Epidinium ecaudatum* extracts do not hydrolyse methyl α-D-glucoside, αα-trehalose or turanose, and have little or no action on sucrose (Bailey, 1958; Bailey *et al.* 1962). The results with the last-named organism have been confirmed in the present work.

In our experiments with sucrose and the epidinia extract, no linkages stable to mild acid hydrolysis

were formed by transferase action, as would have been expected from the action of an α-glucosidase on sucrose. It is concluded that the feeble hydrolysis of sucrose obtained with the epidinia extracts is caused by a trace of a true invertase. This most probably comes from a very slight contamination of the original suspension of epidinia with holotrich protozoa, which are very rich in this enzyme (Howard, 1959).

The hydrolysis of isomaltose by rumen protozoa has so far been studied only in epidinia. Isomaltase, but no maltase activity, was detected in water extracts of these protozoa by Bailey *et al.* (1962). It seems more probable that the slight isomaltase activity found in the subsequent phosphate buffer extracts represents the remaining isomaltase not extracted by water, rather than the activity of the maltase itself.

The specificity of the protozoal maltases is further demonstrated by the results of the experiments with maltotriose. As measured by the liberation of glucose, the epidinia and entodinia preparations were only one-third as active against this substrate as against maltose, and the *D. ruminantium* preparations nearly half as active. However, measurement of both glucose and maltose liberated from maltotriose by the epidinia enzyme indicated that only half of the glucose could have arisen from cleavage of the terminal non-reducing link of maltotriose; the remainder must have come from subsequent hydrolysis of the liberated maltose. It appears therefore that, at least for the epidinia, the maltase can hydrolyse maltotriose at about only one-sixth of the rate at which maltose is hydrolysed.

Experiments with higher maltodextrins could not be carried out with the *D. ruminantium* and entodinia extracts because these contain amylase. It was hoped, by choosing a preparation from epidinia which contained very little amylase, to obtain a valid estimate of the activity of the maltase against maltodextrins. Liberation of glucose from malto-tetra-, -penta-, and -hexa-ose by this preparation was low, and approximately the same as from maltotriose. The presence of maltose and maltotriose in the digests of the penta- and hexasaccharide during the early stages, however, showed that the amylase was interfering substantially in the hydrolysis. It seems certain that, were the amylase absent, the liberation of glucose from the maltodextrins would be even less than was observed. We feel justified in concluding, therefore, that the maltodextrins are not good substrates for the maltase of *Epidinium ecaudatum*.

Further evidence on specificity comes from the results obtained with sugar alcohols as substrates. All three maltases were inactive against maltitol, and the epidinia and entodinia maltases were also inactive against maltotri-itol. The maltase of *D. ruminantium* seemed more versatile than the others; it released glucose from maltotri-itol, and in addition had a slightly greater activity towards maltotriose.

With one exception (the temperature relationships of *D. ruminantium* maltase), the conditions of pH and temperature for maximum activity of these enzymes are not unexpected for organisms living in the rumen. We cannot offer any explanation for the observed stimulation of the epidinia and entodinia enzymes by citrate ions. So far as we are aware, no similar observation has been reported for any other maltase. Another puzzling feature of the results is the discrepancy between the pH optimum found for the entodinia maltase by Abou Akkada & Howard (1960) (pH 5), and that found in the present work (pH 5·7–6·1). This difference can perhaps be partly accounted for by the considerable differences in conditions and methods of assay used in the two sets of experiments. The activity of the epidinia enzyme is very sensitive to temperature changes; at 42° it is less than half as active as at 38°. In contrast, the temperature relationships of the maltase of *D. ruminantium* appear to have no correlation with the physiological conditions under which it acts. Its activity at the usual temperature is less than three-quarters of the maximum, and it appears to be as active at 60° as at 38°.

Under suitable conditions probably all maltases can act as transglucosidases; the site on the acceptor molecule to which glucose is transferred seems to depend on the source of the enzyme. Although in the present work a detailed study of the products of transferase action was possible only with the epidinia enzyme, from the viewpoint of classification the transferase results obtained with all three protozoal enzymes seem of interest. From solutions containing more than 1% of maltose and the epidinia enzyme, two higher sugars, a possible trisaccharide and tetrasaccharide respectively, were isolated. On the evidence from paper-chromatographic studies, the optical rotation, and partial acid hydrolysis of the sugar and its alcohol, it is concluded that the trisaccharide is maltotriose. On paper-chromatographic evidence the other sugar appears to be maltotetraose. Although in our experiments there was good transferase activity in the presence of 1–2% of maltose, many of the results of other workers referred to below were obtained in the presence of 15% of maltose. When this latter concentration of maltose was used with the epidinia enzyme, only the same tri- and tetra-saccharide as before were detected. Our quantitative experiments on the hydrolysis of maltose were carried out at a substrate concentration of 0·18%, which is well below the lowest at which transferase activity could be detected.

On the paper-chromatographic evidence, the entodinia maltase appears to have the same transferring properties as that of the epidinia; substances provisionally identified as maltotriose and maltotetraose were the only new compounds detected. In none of the solutions, from experiments with either epidinia or entodinia enzyme, was there any sign of panose or isomaltose, although in the chromatographic solvent systems used these two sugars could be clearly separated from maltose, maltotriose and maltotetraose, and would have been detected even if present in only trace amounts. The maltase of *D. ruminantium* differs from those of the epidinia and entodinia by producing from maltose in transfer experiments two possible trisaccharides; these have been tentatively identified on paper-chromatographic evidence as maltotriose and panose.

Maltases acting on maltose as both substrate and acceptor produce either maltotriose (if transfer is to the terminal C-4), or panose (if transfer is to the terminal C-6), and isomaltose, if C-6 of the liberated glucose is also able to act as an acceptor. Transfer by maltase to other carbon atoms in the non-reducing glucose unit of maltose, or to any carbon atom in the reducing glucose unit, has not so far been reported. Maltases can therefore be classified according to their ability to transfer to these positions. Three types may be distinguished, involving transfer to C-4 or C-6, or both. Thus several fungal maltases transfer only to C-6 of both glucose and maltose (see Duncan & Manners, 1958, for references to these and other maltases mentioned); *Escherichia coli* and rat-liver maltases transfer only to C-4 of maltose, as does that of *Phaseolus radiatus* (Nigam & Giri, 1960). The maltases of *Cladophora rupestris* and *Tetrahymena pyriformis* (Archibald & Manners, 1959) can transfer to both C-4 and C-6 of maltose, but of these two enzymes only that of *T. pyriformis* can transfer to C-6 of glucose.

Of the three maltases examined in the present work, those of the epidinia and entodinia resemble in transferase properties those of *E. coli*, rat liver and *P. radiatus*, in that they transfer only to C-4 of maltose. The *D. ruminantium* maltase resembles most closely in transferase properties that of *C. rupestris*, in that it can transfer to both C-4 and C-6 of maltose, but not to C-6 of glucose.

The experiments reported here show that there is a close similarity in the properties of the maltases of the epidinia and the entodinia, but that the maltase of *D. ruminantium* differs in important respects from the other two. This distinction might be expected on taxonomic grounds, for *Epidinium* and *Entodinium* are both genera of oligotrich protozoa, and *Dasytricha* belongs to the holotrich group. A further distinction might be made between *D. ruminantium* and the two oligotrichs, in that *Epidinium* and *Entodinium* both swallow and digest starch grains, but *D. ruminantium* does not. The maltases of the oligotrichs therefore would be expected to play a part in converting the food swallowed by the organism into forms suitable for further metabolism. It seems that the maltase of *D. ruminantium* can play no such part in food decomposition. Both of the genera of rumen holotrich protozoa, *Dasytricha* and *Isotricha*, are able to synthesize storage granules of amylopectin when supplies of fermentable carbohydrate are available, and to decompose this material when exogenous foodstuffs become exhausted. However, utilization of this amylopectin can hardly depend on the presence of maltase, since the *Isotricha* species do not produce this enzyme. The part played by maltase in the metabolism of *D. ruminantium* is thus obscure at present.

## SUMMARY

1. Extracts of cells of three species of rumen protozoa, *Dasytricha ruminantium*, *Epidinium ecaudatum* (Crawley) and *Entodinium caudatum* have been prepared, and the properties of some of their carbohydrases studied, with particular reference to maltase.

2. The maltase of *D. ruminantium* has maximum activity at pH 5·5, that of *Epidinium ecaudatum* at pH 6·7, and that of *Entodinium caudatum* at pH 5·7–6·1.

3. The maltases of *Epidinium ecaudatum* and *Entodinium caudatum*, but not that of *D. ruminantium*, are more active in buffer solutions containing citrate ions than in phosphate.

4. The maltases of *Epidinium ecaudatum* and *Entodinium caudatum* are most active at 38°, and their activity is much reduced at temperatures above 40°. The *D. ruminantium* maltase has maximum activity at 50°, and retains moderate activity at 60°.

5. The three enzymes are specific maltases, and do not hydrolyse methyl α-D-glucoside, αα-trehalose, turanose, sucrose or maltitol. Only the *D. ruminantium* enzyme can hydrolyse maltotri-itol. Maltotriose is slowly hydrolysed by all three enzymes.

6. All three enzymes exhibit transferase properties when incubated with 1% (w/v) or higher concentrations of maltose. *Epidinium ecaudatum* and *Entodinium caudatum* transfer glucose to C-4 of maltose; *D. ruminantium* transfers to both C- and C-6 of maltose.

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# The Action of some α-Amylases on Starch Granules

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Many enzymes that can convert starch solution into sugars are known, but it is not known whether all the enzymes that degrade dissolved starch can also attack starch granules. The researches of early workers, which have been reviewed by Reichert (1913), indicated that certain crude α-amylases could slowly hydrolyse raw starch. The view is still held that α-amylase is responsible for the breakdown of intact starch granules (Kneen, Beckord & Sandstedt, 1941), but it remains to be explained why the speed of the hydrolysis of raw starch observed *in vitro* is so much slower than *in vivo*. In the present paper the action is described of both crude and crystalline α-amylase from various sources on cereal, tuber and protozoal starch. Some factors that are of importance for the rapid digestion of starch granules are discussed.

## MATERIALS AND METHODS

*Tuber starch.* Starch was prepared from potatoes as described by Schoch (1957). Soluble starch (Lintner) was supplied by Hopkin and Williams Ltd.

*Cereal starch.* Waxy-maize starch was prepared by the method of Schoch (1957) from hand-sorted single-cross Tapicorn seed kindly given by the Bear Hybrid Corn Co., Ill., U.S.A. Maize starch was prepared similarly. Rice starch was prepared as described by Whelan (1955).

*Protozoal starch.* Starch was prepared from the rumen ciliates *Holotricha* spp. as described by Mould & Thomas (1958), the deproteinization with organic solvent being omitted. Maltose hydrate was supplied by Thomas Kerfoot Ltd., Vale of Bardsley, Lancs. Sodium deoxycholate was Laboratory Reagent grade (British Drug Houses Ltd.).

*Amylases.* Saliva was collected from several people and centrifuged after 1 hr. to remove mucins. Salivary α-amylase was purified by the method of Fischer & Stein (1961). Human salivary α-amylase (crystalline) was a gift from Dr E. A. Stein. A crude α-amylase from pig pancreas was prepared by grinding the minced pancreas (148 g.) with borate buffer (60 ml.) at pH 8·5. The mixture was allowed to stand at 0° for 1 hr. with occasional stirring, and then was filtered through gauze. The extract was clarified by centrifuging at 80 000*g* and an acetone-dried powder was prepared from the supernatant. A portion (489 mg.) of this powder was extracted with a solution (5 ml.) containing 0·1 M-sodium chloride, 0·01 M-calcium acetate and 0·02 M-tris–maleate buffer, pH 6·9. The insoluble material was removed by centrifuging and the clear extract (7500 units/ml.) was used as the source of crude α-amylase. Pig pancreatic α-amylase (crystalline) was purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A. A crude *Bacillus subtilis* α-amylase was prepared from the extracellular α-amylase produced in a broth culture of *B. subtilis*. The broth was clarified by centrifuging and dialysed against distilled water containing 1 mM-$Ca^{2+}$ ion to remove reducing substances. The dialysed broth (400 units/ml.) was used as the source of crude α-amylase. *Bacillus subtilis* α-amylase (crystalline) was a gift from Daiwa Biochemical Products Co. Ltd., Osaka, Japan. A crude *Aspergillus oryzae* α-amylase was prepared from Clarase 900 supplied by the Takamine Laboratory Inc., N.J., U.S.A. Clarase 900 (6 g.) was stirred slowly with water (37·5 ml.) at 2° overnight. The undissolved residue