The Enzymic Degradation of Starch by Holotrich Protozoa from Sheep Rumen

By D. L. MOULD and GWEN J. THOMAS*
Rowett Research Institute, Bucksburn, Aberdeenshire

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It has been shown that the holotrich ciliates found in the rumen of sheep fed on a starch-free diet can store large amounts of iodophilic granules (Masson & Oxford, 1951). This material was identified as starch rather than glycogen since it gave an insoluble iodine complex under the conditions described by Pucher, Leavenworth & Vickery (1948). Further evidence was given by Forsyth & Hirst (1953) who proved by methylation studies that the storage material was an amylopectin having an average chain length of 25 glucose units. Some experiments with β-amylase and with the debranching enzyme R-enzyme, to be described below, also point to the similarity between this storage material and potato amylopectin.

During the course of experiments designed to distinguish between several mechanisms which might be involved in the synthesis of amylopectin by the protozoan organisms, it became apparent that the search for the synthesizing enzyme was being complicated by the presence of a highly active amylase in the cell extract. The purification of this amylase, and its separation from the other carbohydrases found in the Protozoa, are described in this paper. During fractionation by salt-precipitation methods or by the use of specific absorbents a certain amount of activity was lost (cf. Bernfeld, 1955). Since differences between the isoelectric points of amylases and invertases exist (Dieu, 1947; Bernfeld, Duckert & Fischer, 1950; Fischer, Kohtès & Fellig, 1951; Fischer & de Montmollin, 1951*a*) it was thought that fractionation of the mixture of amylase, maltase, β-glucosidase, invertase and the starch-synthesizing enzyme present in the cell contents of the Protozoa might be achieved by use of the methods of preparative zone electrophoresis (Tiselius & Flodin, 1953). The method has been partially successful, with the result that a reversible phosphorolytic enzyme system similar to that existing in the higher plants and animals has been found to operate in the protozoan cell. Also of interest is the separation of three specific amylases present in aqueous extracts of holotrich Protozoa. The amylolytic action of two of the amylases has been determined, and an approximate estimate of their electrophoretic mobility and isoelectric points has been made.

* Present address: Lister Institute for Preventive Medicine, London.

MATERIALS AND METHODS

Preparation of holotrich material

Phosphate–acetate buffer. The composition of this was (%, w/v): NaCl 0·5, sodium acetate 0·13, KH_2PO_4 0·03, K_2HPO_4 0·10, $MgSO_4,7H_2O$ 0·01. The solution was adjusted to pH 7·2 with dilute NaOH.

Citrate buffer. Sodium citrate (0·2 M) was adjusted to pH 5·5 with 5 N-NaOH.

Separation of holotrich ciliates from rumen fluid. The method was essentially that of Heald & Oxford (1953). A sample of rumen contents (usually 1 l.) was taken with tube and suction bulb 2 hr. after the sheep had been fed. The sample was strained through six layers of surgical gauze, and allowed to stand at 39° for 1 hr. Half the supernatant was sucked off, and the bottom layer, which contained Protozoa and debris, was put into a separating funnel. Glucose dissolved in phosphate–acetate buffer was added to give a final concentration of 0·75%, and the funnel was placed in the incubator. The white bottom layer, settling during fermentation of glucose, was withdrawn at intervals into boiling tubes filled with the buffer at pH 7·2. Several washings by decantation were required to remove the scum and debris which rose to the surface with the gas produced by the holotrichs. In those cases where it was intended to test the Protozoa for phosphorylase activity the final washings were carried out with citrate buffer. The Protozoa were then transferred into screw-capped bottles and stored at −20° until required.

Separation of Isotricha *and* Dasytricha. Microscopic observation of the ciliates collected as above showed that the rumen holotrichs *Dasytricha rumiantium*, *Isotricha prostoma* and *I. intestinalis* were invariably present, although the proportion of *Isotricha* and *Dasytricha* varied to a marked extent. *Isotricha* and *Dasytricha* were separated by a modification of the technique of Gutierrez (1955). The Protozoa which had been drawn off the base of the separating funnel were divided into six portions, each of which was poured on top of separate liquid columns containing clarified rumen liquor (38 ml.), phosphate–acetate buffer (15 ml.) and glycerol (3 ml.). Protozoa which settled during the first 10 min. were the heavier *Isotricha*, and these were drawn off and washed with buffer in the usual manner. *Dasytricha*, which remained suspended in the column, were deposited during centrifuging and, after several washings with buffer, were stored at −20°. Overloading of the columns led to poor separation of the Protozoa since some *Dasytricha* were brought down with the *Isotricha*. Portions

of each fraction drawn off the columns were examined under the microscope, and those tubes containing only *Isotricha* were kept, and their contents pooled. Gutierrez's method of isolating the *Dasytricha* which remained as streaks on the separating funnel after the rumen contents had been drained out was not adopted as the yield obtained in this way was too low.

Counting the Protozoa. Counts of the Protozoa suspended in buffer–glycerol (1:1) were made in a cell described by Boyne, Eadie & Raitt (1957).

Preparation of cell-free filtrates. Protozoa which had been stored at −20° were quickly brought to room temperature by immersing the bottles in water at 25°. This caused the Protozoa to burst, thus liberating the starch granules. The cell debris and starch were removed by centrifuging, and the supernatant was dialysed in cellophan for 48 hr. at 2° with stirring against distilled water. In this way the enzymes were obtained free from starch dextrins and various sugars. The extract was freeze-dried, and could be stored for 6 months without loss in activity of the amylases.

Preparation of substrates and enzymes

Amylose. This was prepared from potato starch by the $Al(OH)_3$–thymol procedure of Hobson, Pirt, Whelan & Peat (1951).

Amylopectin. This was prepared by fractionation of thymol–amylopectin with the methanol procedure of Hobson *et al.* (1951).

Holotrich starch. The starch granules were liberated from the ciliates as described above. Much of the cell debris was removed by light centrifuging, which left the starch in suspension. The starch was further purified by the technique of Sevag (1934). A solution of the starch was shaken with $CHCl_3$ and amyl alcohol for 24 hr. in a centrifuge tube. The mixture was centrifuged for 30 min. and the top layer containing the starch was removed. The starch was precipitated with 3 vol. of ethanol. The purity as determined by acid hydrolysis and estimation of reducing sugar was 97%.

Grass fructan. Italian ryegrass was cut before the flowering stage and air-dried at 100°. The milled grass was extracted in a Soxhlet thimble with 85% (v/v) ethanol for 8 hr. This treatment removed all pigments, hexoses, sucrose, raffinose and some short-chain polyfructosans. The grass residue was shaken with water overnight, filtered on a Büchner funnel, and the filtrate treated with 4 vol. of ethanol. The precipitated fructan was dissolved in water and desalted by passage through ion-exchange columns packed with Amberlite IR-120 (H) and -4B (OH). The deionized fructan was again precipitated with ethanol, and dried over P_2O_5 *in vacuo* at room temperature. The product had $[\alpha]_D$ −42°. ($R_F = 0$ in propanol–ethyl acetate–water solvent mixture, 6:1:3, by vol.)

Salivary α-amylase. The method of preparation of Meyer, Fischer, Staub & Bernfeld (1948) was followed to the stage before crystallization.

R-enzyme. This was prepared from broad beans as described by Hobson, Whelan & Peat (1951).

β-Amylase. Pure β-amylase was prepared from soya beans by the method of Peat, Pirt & Whelan (1952).

Measurement of enzyme activity

Amylase. A solution of potato amylose (0·2%) was prepared by dispersing amylose wetted with ethanol in 0·1 N-NaOH and heating at 100° for 5 min. After it had been cooled and neutralized with 0·1 N-H_2SO_4, the solution was appropriately diluted. The volume added to the digest mixture was arranged to bring the final concentration of amylose to 1 mg./ml. In most cases a 6 ml. digest was prepared, containing 1 ml. of 0·2 M-citrate buffer (pH 5·5). The amount of enzyme added was such that the fall in light absorption of the polysaccharide–iodine solution after 15 min. at 35° was not more than 7 scale units on the EEL colorimeter (Evans Electroselenium Ltd.) with 1·3 cm. diam. cells and Ilford filter no. 608 (max. transmission 680 mμ) when a sample (1 ml.) of the digest was withdrawn and made up to 100 ml. with I_2 (2 mg./100 ml.) and KI (20 mg./100 ml.) for measurement of EV (Hobson & Macpherson, 1952); EV is the light-absorption measured on the EEL colorimeter. One activity unit was defined as the amount of amylase which produced a fall in EV of one colorimeter scale unit under the above conditions.

β-Glucosidase, invertase and maltase. Digests of total volume 6 ml. contained 1 ml. of citrate buffer (pH 5·5) and the concentration of substrate was usually 0·3% for maltose and cellobiose, 2% for sucrose and 1·5% for fructan. Portions (1 ml.) were withdrawn at intervals for the determination of reducing power with the Somogyi (1945) reagent. The increase in reducing power during the incubation was a measure of the activity of the enzyme.

Phosphorylase. The activity was determined by measuring the amount of glucose 1-phosphate formed in a digest containing amylose and phosphate. Amylose (140 mg.) was dissolved by heating in 0·1 N-NaOH and brought to neutrality with N-H_2SO_4. Ammonium molybdate (8·3%, w/v; 8 ml.) and 0·002 M-$HgCl_2$ (0·6 ml.) were added and the solution was made up to 50 ml. with phosphate buffer (30 ml.). The phosphate buffer contained KH_2PO_4 (13·617 g.) and Na_2HPO_4 (35·995 g.) in 1 l., giving a concentration of 6·5 mg. of P/ml.

Digests containing amylose solution (3 ml.) and phosphate buffer (2 ml.) were prepared and brought to 35°. Enzyme samples (2 ml.) were then added and the digest was incubated at 35° for 20 hr. Excess of inorganic phosphate was removed by precipitation with magnesia reagent and centrifuging. The magnesia reagent contained $MgCl_2$ (14 g.), NH_4Cl (17 g.) and strong aq. NH_3 soln. (sp. gr. 0·880; 30 ml.) in 250 ml. of solution. After centrifuging, determinations of free inorganic phosphate and of 7 min.-hydrolysable phosphate were made by the method of Allen (1940). The difference between these represented the amount of glucose 1-phosphate present, and this was related to the activity of the enzyme.

Zone electrophoresis

Zone electrophoresis was carried out in starch columns according to the method of Flodin & Porath (1954) (see also Flodin & Kupke, 1956). The holotrich amylases were strongly adsorbed to cellulose and cellulose derivatives, as was discovered during preliminary studies of the mobility of the amylases on filter-paper strips or cellulose columns. No tailing of the enzyme preparations was, however, observed in starch columns and there was full recovery of enzyme activity in the eluate. Crystalline salivary α-amylase was strongly adsorbed to the starch columns. This may possibly be due to an insolubility effect and surface denaturation of the pure preparation. A preparation of *Streptococcus bovis* amylase appeared to be unaffected by elution through a packed-starch column, but the activity of

the preparation was too low for a satisfactory investigation of the trailing edge of the eluted zone.

Apparatus. Commercial potato starch was sieved and the fraction passing through a 300-mesh sieve was used as the packing material for the column. It was washed and treated with buffer as described by Flodin & Porath (1954). The column consisted of a glass tube 55 cm. long × 3 cm. diam. with an outer cooling jacket along its whole length, through which water from a thermostatic bath (10–25°) was circulated. The starch packing was supported above the glass filter at the base of the column by a close-packed glass-wool plug. The total length of the packed column was 48·5 cm., the effective cross-sectional area 2·4 cm.^{2} and the ratio starch vol./mobile liquid vol. was 2·0. The hold-up volume was 116 ml. The general construction of the apparatus was, with a few modifications, in principle the same as that described by Flodin & Porath (1954). Large silver–silver chloride electrodes were used sufficient for a total passage of 8000 C. A p.d. of 300 V was applied from a stabilized high-tension supply, and the duration of electrophoresis was 18–20 hr.

An appreciable electro-endosmotic flow is developed through a starch medium in the presence of phosphate buffers (Kunkel & Slater, 1952). For reproducible electrophoretic movement of zones in the column it was found necessary to leave the electrode vessels open and allow a head of buffer solution to be formed in the cathode vessel which could then act as a hydrostatic counterbalance for the electro-osmotic flow. The requisite difference in levels between the two electrode vessels at the commencement of electrophoresis for any set of experimental conditions could quickly be established by experience, e.g. a difference of 12·1 cm. was required with phosphate buffer, pH 7·0, *I* 0·05, electrophoresis current 13·7 mA and column temp. 20°. Volume changes due to electrode reactions occur slowly throughout the duration of electrophoresis but the auto-regulated head is able to adjust itself to these changes and remains constant, although the level in both electrode chambers may fall. Since the electrode vessels themselves were of large volume it was also advantageous to keep the temperature of the surroundings reasonably constant. All electrophoresis was carried out at an ambient temperature of 16–20°. The current remained constant to within 1% in 24 hr. under these conditions. The anode was always connected to the bottom of the packed column. At the end of the electrophoresis the starch column was eluted with buffer at a flow rate of 15 ml./hr. and 4 ml. fractions were collected.

Buffer solutions. Sodium phosphate buffer, pH 7·0, *I* 0·05, was used throughout. Stock solutions of 0·2 M-Na_2HPO_4 (135 ml.) and 0·2 M-NaH_2PO_4 (95 ml.) were diluted to 2 l.

Preparation of enzyme solutions. Samples (100 mg.) of freeze-dried dialysed aqueous extracts of holotrich organisms were dissolved in phosphate buffer (2 ml.) and centrifuged at 14 000 *g* for 15 min. The clear supernatant solution, usually brown or straw-coloured, was applied to the column to form the initial zone (1·5–1·8 ml.). Samples of the supernatant solution were also taken for determination of enzyme activity.

Determination of enzyme activity. Amylase activity was determined by a similar method to that described above. The distribution of enzyme activity in the eluate fractions after electrophoresis was tested by taking samples (0·2–0·5 ml.) from each fraction according to the total activity applied to the column. In order to keep the sample volumes small and thereby conserve the amylase fractions the digests were incubated for 1 hr. at 35°. The digests consisted of 2 ml. of amylose solution (2 mg./ml.), 1·8 ml. of acetic acid–sodium acetate buffer, pH 4·8, and 0·2 ml. of enzyme solution. Measurement of EV was by the usual procedure (Hobson & Macpherson, 1952) and expressed in the scale units of an EEL colorimeter. Approximate estimates of total activities of the enzyme solutions were made by summation over the elution curves, assuming a linear relation in EV with enzyme concentration and one activity unit being defined as the amount of amylase which produced an EV of one colorimeter scale unit under these digest conditions.

Invertase, phosphorylase and other enzyme activities were determined as described above.

Dry-weight determinations. In certain fractionations, the distribution of total dry weight in the eluate from the starch columns after electrophoresis was measured. Eluate fractions (2 ml.) of known weight were collected in small weighing bottles and freeze-dried. Drying was completed to constant weight *in vacuo* over conc. H_2SO_4.

Ovalbumin preparation. A preparation of ovalbumin was used as a comparison material for estimating the electrophoretic mobility of the enzymes in the column. It was prepared from egg white by half-saturated $(NH_4)_2SO_4$ precipitation (Warner, 1954), and was recrystallized twice, dialysed and freeze-dried. The preparation contained both ovalbumin and conalbumin and separated into two zones of quite distinct mobility during zone electrophoresis in phosphate buffer on the starch column. The distribution of the protein in the eluate was observed by measuring the light-absorption of the fractions at 280 mμ in a 1 cm. cuvette on a Beckman DU quartz spectrometer.

Boundary electrophoresis. Analyses of the ovalbumin–conalbumin mixture and a holotrich enzyme preparation were made in phosphate buffer, pH 7·0, *I* 0·1, under standard procedure in a Hilger boundary electrophoresis apparatus by Dr E. I. McDougall.

RESULTS

Action of holotrich extract on amylose

When the cell-free extract was allowed to act on potato amylose the digest rapidly became achroic. The iodine stain of portions of the digest was tested at intervals, and the colour changes from blue through purple and red to orange were typical of those which occur during α-amylolysis. The products of the reaction, identified on a paper chromatogram, were glucose, maltose and maltotriose. This distinguished the enzyme from β-amylase, which would have produced maltose only. The relationship between absorption value and percentage apparent conversion into maltose was also typical of an α-amylase, the conversion at 10% of the original absorption value being 18%, and 30% at the achroic point.

The optimum pH and temperature of action of the enzyme were determined, and found to be pH 5·5 and 50° respectively. The digests prepared

for the experiments were similar to the standard digests described above but sodium acetate–veronal buffer was used for the optimum pH experiment.

Effect of chloride and calcium ions on the amylase activity

Digests were set up containing $CaCl_2$ and NaCl to give a concentration of 0·0167 M. The enzyme was not activated by either salt, nor by 0·1 M-$MgSO_4$. Thus the amylase may be contrasted with salivary α-amylase, which is activated by 0·001 M-NaCl, and with malt α-amylase, which requires Ca^{2+} ions (Schwimmer & Balls, 1949; see Bernfeld, 1951).

Amylolytic degradation of holotrich starch

The reaction between holotrich extract and potato starch continued until the starch was completely converted into glucose. Holotrich starch was also completely degraded. This points to the presence in the extract of an amylo-1:6-glucosidase, and also a maltase since amylases have no action on maltose. Attempts were made to separate maltase from amylase by fractionation with ethanol, and with ammonium sulphate, but no substantial increase in the ratio amylase: maltase was obtained. There was, however, a large variation in the maltase activity of holotrich extracts made from rumen samples taken at different times.

When potato amylopectin and holotrich starch were incubated with salivary α-amylase, the reaction was arrested at 68 % conversion into maltose (Table 1). This limit was raised to 100 % if the amylase was allowed to act together with the debranching enzyme R-enzyme. The results in Table 1 show that holotrich starch and potato amylopectin are attacked in exactly the same way by R-enzyme and α-amylase. The simultaneous action of the two enzymes proceeds to a further extent than does the successive action, showing that R-enzyme cannot fully debranch amylopectin unless some of the α-1:4-glucosidic linkages between the branch linkages are hydrolysed by α-amylase. The characteristic increase in the iodine stain of amylopectin during incubation with R-enzyme was also observed when R-enzyme acted on holotrich starch.

Pure β-amylase prepared from soya beans hydrolysed holotrich starch to the extent of 51·5 % as maltose. The same result was obtained with starch prepared from *Isotricha*, and with starch from *Dasytricha*. Starch was also extracted from holotrich Protozoa which had been allowed to store polysaccharide at a lower temperature (25°) than usual, thus producing an abnormal appearance described by Eadie & Oxford (1955) in which the starch granules clump together in the middle of the cell. This starch was converted into maltose to the extent of 57 %. This 'abnormal' starch was similar to normal holotrich starch in other tests. Both starches exhibited behaviour similar to amylopectin during ultrafiltration in collodion membranes (Mould & Synge, 1954). After chromatography in formamide (Nussenbaum, 1951) both holotrich starches stained red with iodine and remained at the origin with no streaking. Amylose and soluble starch were used as controls. The wavelength of maximum absorption of the iodine–holotrich polysaccharide complex was also the same (540 mμ).

The simultaneous action of R-enzyme and β-amylase on holotrich starch gave a conversion into maltose of 100 %. Thus R-enzyme can hydrolyse all the branch linkages of holotrich starch after they have been exposed by β-amylase action. In this respect the starch differs from potato amylopectin, which has never been completely hydrolysed by R-enzyme plus β-amylase action, owing, it is thought, to the presence of phosphate groups which hinder the access of R-enzyme to some of the branch linkages (Peat, Whelan & Thomas, 1956). Waxy-maize starch, which has a negligible phosphorus content, is completely converted into maltose by the simultaneous action of R-enzyme and β-amylase (Thomas, 1952).

Electrophoretic separation of holotrich amylases

In preliminary experiments for the determination of optimum conditions for fractionation of the enzymes, zone electrophoresis was carried out in veronal buffer, pH 8·6 (*I* 0·1 or 0·05), and at

Table 1. *Enzymic degradation of amylopectin*

For conditions see text. The following abbreviations are used: α, salivary α-amylase; β, soya-bean β-amylase; R, R-enzyme; R, α, successive action of R and α; R + α, simultaneous action of R and α; R + β, simultaneous action of R and β.

	Apparent conversion into maltose (%)						
	Holotrich starch				Potato amylopectin		
Time (hr.)	α	R, α	R + α	R + β	α	R, α	R + α
2	32·8	65·8	67·7	63·2	29·4	71·9	73·9
8	42·2	73·8	81·9	81·9	51·2	75·9	81·3
22	60·6	78·0	86·1	88·4	61·9	82·5	85·3
70	68·3	86·5	98·1	101·5	64·5	87·8	101·2
99	66·4	86·8	99·4	—	68·1	—	101·8

temperatures 20–26° or 12–14°. Two well-defined zones of amylase activity were observed with an overlap of an invertase zone with the faster-moving zone. Recovery of amylase activity from the column was, however, only 50–70%, presumably due to the alkaline conditions. With phosphate buffer, pH 7·0 (I 0·05), and a column temperature 20°, total recovery of enzyme activity was achieved with good separation of the zones and these conditions were used throughout. With an applied p.d. of 300 v the current through the column was 13–14 mA and the time of electrophoresis 20 hr.

The distribution of amylase and invertase activity in the eluate fractions is shown in Fig. 1*a*. There are two well-separated peaks of amylase activity distinguished subsequently as A_D and A_I for the fast- and slow-moving components respectively. The invertase peak always overlapped the leading edge of A_D and has never been completely resolved. The proportion of total activity between the two amylase peaks varied considerably for different enzyme-extract preparations. The ratio A_D/A_I varied from 1·1 to 2·5 over eight separate preparations and in two extreme cases was 6·1 and 0·6. The actual height of the peaks depends on the sample volume and the original loading of the column.

The eluate fractions containing maximum amylase activity were pooled, freeze-dried and stored at 2°. The dried material was redissolved in a small volume of water and, after dialysis against phosphate buffer, reapplied to the column and electrophoresis was repeated under identical conditions. The enzyme activity was very much reduced after this procedure but it could be shown (Fig. 1*b*, *c*) that the enzyme fractions retained their characteristic mobility although there was extensive tailing of the slow-moving peak A_I.

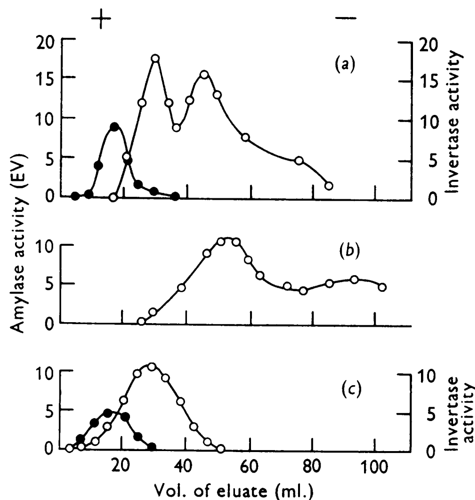

Fig. 1. Distribution of amylase and invertase activity in eluate from column after zone electrophoresis. Phosphate buffer, pH 7·0, I 0·05. (*a*) Mixed holotrich extract. (*b*) Repeat electrophoresis of material in slow-moving peak. (*c*) Repeat electrophoresis of material in fast-moving peak. ○, Amylase activity; ●, invertase activity.

A detailed examination of the total loss of activity of the fractionated enzymes during freeze-drying and dialysis was made. There was a 20% loss in invertase activity on freeze-drying in concentrated phosphate and a further loss amounting to 25% on prolonged dialysis against distilled water. The loss in activity on freeze-drying the amylase fractions could be as high as 40%, but they are fairly stable to extended dialysis against phosphate buffer (pH 7·0, I 0·02). Dialysis of the amylase solutions in the presence of 0·01 M-$CaCl_2$ gave similar results. Amylase A_D would appear to be more stable than A_I, which is in agreement with the general observed behaviour of fractionated and stored material. The enzyme stability is likely to be affected by partial purification and the adsorption tailing observed in the repeat electrophoresis of amylase A_I might be explained as arising from the deposition of insoluble enzyme on the surface of the supporting media. The unfractionated enzyme preparation from holotrich Protozoa was very stable towards dialysis and freeze-drying.

The amylolytic behaviour of the enzymes was investigated with the various peak eluate fractions from the column in the presence of phosphate buffer immediately after electrophoresis.

In order to assay further the degree of purification of the enzymes the distribution of total dry matter in the eluate fractions after electrophoresis was examined (Fig. 2). The dry solid content of the buffer in the column was 3·5 mg./ml. The two peaks of amylase activity lie on either side of quite a sharply defined peak of maximum dry solids. Amylase A_D is associated with the peak more than amylase A_I, which is apparently enriched. This is in agreement with the observed higher stability of the former enzyme during freeze-drying, storage

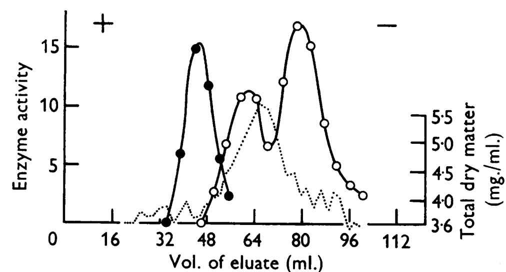

Fig. 2. Distribution of total dry matter and enzyme activity in eluate from column after zone electrophoresis of mixed holotrich extract. ○, Amylase activity; ●, invertase activity.

and dialysis. The dry solid content across the invertase peak is very low and the faster-moving portion of this peak must contain very enriched enzyme.

A rough estimation of the purification factor can be made from the data displayed in Fig. 2. The original activity of the invertase/mg., expressed in arbitrary units (see Methods), was 22. Assuming collection and pooling of the invertase fractions uncontaminated with amylase and no loss on freeze-drying and dialysis, the activity/mg. of the preparation would have been 656. The activity of the amylase (both types)/mg. in the original extract is 32. If fractions covering the whole A_I peak were collected and pooled, again assuming no loss of activity, the activity/mg. of the A_I preparation would have been 83.

Electrophoretic behaviour of amylases from Dasytricha *and* Isotricha

On the hypothesis that the enzymes A_I and A_D might be specific to either of the two types of Protozoa present, or that the ratio A_D/A_I might be specific to a particular organism, aqueous enzyme extracts from single organism preparations were subjected to zone electrophoresis.

The electrophoretic distribution of amylase activity due to *Isotricha* (Fig. 3) shows a small A_D component only, compared with A_I. During the sedimentation of the Protozoa from a suspension of filtered rumen contents after the addition of glucose, the larger *Isotricha* are deposited first, so that in normal preparations of mixed Protozoa it is highly probable that there will be a preponderance of *Isotricha* in the mixture. Owing also to the preferential sedimentation of *Isotricha*, there are difficulties in estimating visually the true distribution of organisms in the preparation (Boyne *et al.* 1957). If A_I is associated with *Isotricha* this would account for an occasional tendency in the various enzyme preparations to obtain a very high proportion of A_I to A_D.

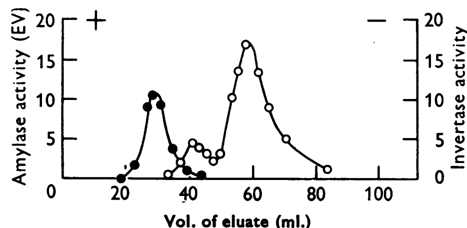

Fig. 3. Distribution of enzyme activity in eluate from column after zone electrophoresis of *Isotricha* extract. ○, Amylase activity; ●, invertase activity.

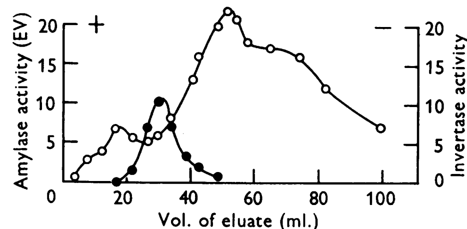

Fig. 4. Distribution of enzyme activity in eluate from column after zone electrophoresis of *Dasytricha* extract. ○, Amylase activity; ●, invertase activity.

The electrophoretic distribution of amylase activity due to *Dasytricha* (Fig. 4) shows a small peak of very high mobility (A_P) and a peak of fast-moving amylse (A_D) overlapped by invertase, with pronounced trailing. There is little doubt that the high value of EV still present at the end of elution of the column is indicative of adsorption, and it is possible that a small peak of slow-moving A_I is superimposed on this tail. No adsorption of the unfractionated *Dasytricha* enzyme extract during trial elution through the column was observed.

Electrophoretic mobility of enzymes. Unless the column conditions are standardized it is impracticable to determine absolute mobilities by measurements of migration. It is possible, however, to compare the unknown mobilities with that of a standard material, and the ovalbumin preparation was used for this purpose.

A comparative zone-electrophoretic fractionation of holotrich enzyme extract and ovalbumin in phosphate buffer, pH 7·0, I 0·05, at 20° is shown in Fig. 5. Boundary-electrophoresis determinations had to be made at I 0·1. In a corresponding fractionation on the column in phosphate buffer, pH 7·0, I 0·1, at 10° poorer separation was evident and also a change in the relative mobilities of the peaks.

The estimated mobilities of ovalbumin and conalbumin in boundary electrophoresis experiments made by Dr E. I. MacDougall were $5{\cdot}50 \times 10^{-5}$

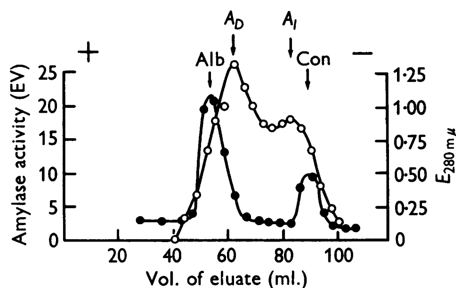

Fig. 5. Column-electrophoresis eluate of mixed holotrich extract and ovalbumin preparation. Phosphate buffer, pH 7, I 0·05, at 20°. ○, Amylase activity; ●, optical density at 280 mμ. (Alb. = albumin; Con. = conalbumin).

and $1\cdot48 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ respectively in phosphate buffer, pH 6·94, I 0·1, at 0·6°. The holotrich enzyme extract under the same conditions gave two peaks $4\cdot23 \times 10^{-5}$ and $3\cdot31 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$. Assuming a proportional increase in the mobilities of the various components with temperature it was inferred that the mobilities of A_D and A_I are about $5\cdot4 \times 10^{-5}$ and $1\cdot3 \times 10^{-5}$ cm.^{2}v^{-1} sec. respectively in phosphate buffer, pH 7·0, I 0·1, at 0·6°.

Isoelectric points. A series of zone-electrophoretic separations of a mixture of holotrich extract and ovalbumin preparation were carried out at pH 7·00, 5·96, 5·04 (phosphate buffer, I 0·05) and pH 4·24 (acetate buffer, I 0·05). The eluate fractions were tested for amylase and invertase activity and for the presence of ovalbumin and the position of the zones located. The difference between the hold-up volume of the column and the elution volume of a peak was then assumed to be proportional to the electrophoretic mobility of the migrating substance under the particular column conditions. In order to eliminate approximately the different field conditions and electroendosmotic flow resulting from each buffer change, the mobilities of the enzymes were intrapolated from established values for the mobility of ovalbumin and conalbumin under free solution conditions. These electrophoretic mobilities are unlikely to be the same as under the conditions during electrophoresis on a packed-starch column, and will have no precise quantitative significance, but are valid for determining the conditions for zero mobility. The change in mobility with pH is shown in Fig. 6. No amylase activity was detected at pH 4·24. The isoelectric points of invertase and A_I were estimated as 4·15 and 5·40 respectively.

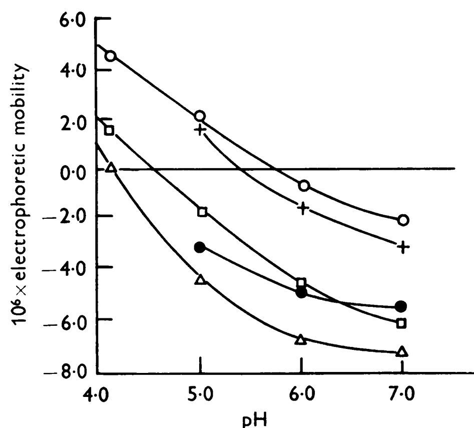

Fig. 6. Change in electrophoretic mobility with pH. See text. ○, Conalbumin; □, ovalbumin; +, amylase A_I; ●, amylase A_D; △, invertase.

Amylolytic properties of the amylases A_D and A_I

The reaction of these enzymes with amylose proceeded until the apparent conversion into maltose was 88 %, and thereafter amylase A_D continued slowly to hydrolyse maltose and maltotriose. The electrophoretic separation of some of the enzymes in an extract of *Dasytricha* is shown in Fig. 7. It can be seen that maltase activity has not been separated from amylase during electrophoresis of an extract of *Dasytricha.* In extracts of *Dasytricha* and *Isotricha* of equal amylase activity it was also shown that the maltase activity of the *Dasytricha* extract was 20 times that of the *Isotricha* and that the *Dasytricha* extract was 6 times more active towards maltotriose. Thus it can be expected that the amylase A_D will hydrolyse amylose completely to glucose, whereas the action of A_I is arrested when amylose has been converted into maltose and maltotriose. Counts of the organisms present in the *Isotricha* and *Dasytricha* preparations are given in Table 2. The low maltase activity apparent in the *Isotricha* extract may be due to the presence of *Dasytricha,* although the activity seemed higher after hydrolysis for 24 hr.

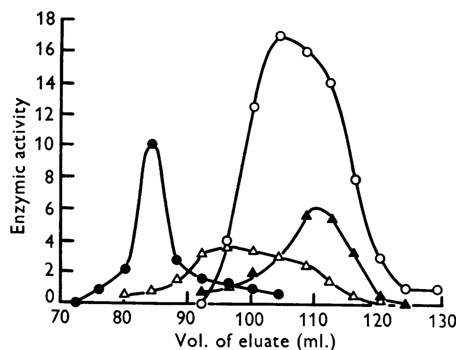

Fig. 7. Distribution of enzymic activity in column-electrophoresis eluate of *Dasytricha* extract. ●, Invertase; △, cellobiase; ○, amylase; ▲, maltase.

Table 2. *Count of* Isotricha *and* Dasytricha *present in purified preparations*

Protozoa were suspended in a 1:1 mixture of phosphate-acetate buffer and glycerol. A 1 ml. sample was placed in a counting cell and the contents of 25–30 squares were counted.

	Number of cells	
Preparation	*Isotricha*	*Dasytricha*
Isotricha	243	4
	242	3
	127	3
Dasytricha	6	736
	4	740
	2	583

than would be expected in relation to the proportion of organisms present.

The optimum temperature for the action of A_D and A_I on amylose was 55°. The optimum pH of action of A_I was 4·8. The activity curve for A_D showed two peaks, at pH 5·0 and 6·0 respectively (Fig. 8). Other amylases which show two maxima in their activity–pH curves are soya-bean α-amylase, malt α-amylase and pig pancreatic α-amylase.

It was of interest to know how the amylase activity per cell of *Dasytricha* compared with that of the *Isotricha*. For this purpose the holotrichs were separated in the usual manner, suspended in buffer, and divided into three portions. One portion was mixed with an equal volume of glycerol, and counts of the Protozoa were made in a 1 ml. counting chamber. The number of Protozoa in 25 squares taken at random from a possible 200 were counted. Another portion of the Protozoa in suspension were burst by freezing and thawing, and, since this procedure is not completely effective for the *Dasytricha*, the suspension was also ground in a glass tissue-crusher. The enzymic activities in the filtrates were then determined. The third portion of each suspension was dried in an oven at 100° overnight. Thus the amylase activity and dry weight of a given number of Protozoa were known. It was found that a single *Isotricha* contained 10·5 times the amylase activity of *Dasytricha*. Since by calculation an *Isotricha* is on average 9·1 times heavier than a *Dasytricha*, it follows that the amylase activity/unit weight is almost identical for the two species.

Action of extracts of Dasytricha *and* Isotricha *on disaccharides and polysaccharides*

The results for cellobiose and maltose are shown in Fig. 9. *Dasytricha* enzyme shows a far greater activity towards cellobiose than does the *Isotricha* extract. This compares with the result of Gutierrez (1955), who found that washed suspensions of *Isotricha* did not ferment cellobiose, whereas *Dasytricha* fermented cellobiose at a rate comparable with that for glucose. Dr G. Halliwell has tested a similar extract by his method for determining cellulase and found no attack on cellulose powder or hydrocellulose (Halliwell, 1957).

The results for maltose and maltotriose have already been discussed. Although the *Dasytricha* can hydrolyse maltose, it is not converted into storage polysaccharide. This is in contrast with the other hydrolysable carbohydrates sucrose, raffinose, inulin and bacterial levan, which can all be rapidly converted into storage polysaccharide by the holotrichs (Masson & Oxford, 1951).

The hydrolysis of sucrose and grass fructan by the holotrich extracts was measured by following the increase in reducing power of standard activity digests. For equal weights of dried Protozoa, the activity of the *Dasytricha* towards sucrose is 1·2 times that of the *Isotricha*. The corresponding value for grass fructan is 1·6.

Phosphorylase activity of holotrich Protozoa

The presence of amylase in the extracts of holotrich Protozoa has the effect of masking the system responsible for the synthesis of polysaccharide firstly by destroying the 'primer' necessary before synthesis can commence, and secondly by hydrolysing any polysaccharide that is synthesized. A search was therefore made for an inhibitor which

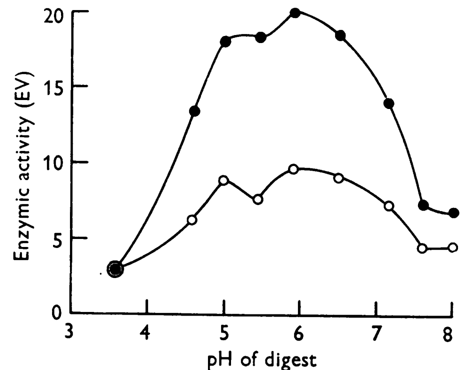

Fig. 8. Effect of pH on amylase A_D activity. ○, Incubated at 35° for 1 hr.; ●, incubated at 35° for 3 hr.

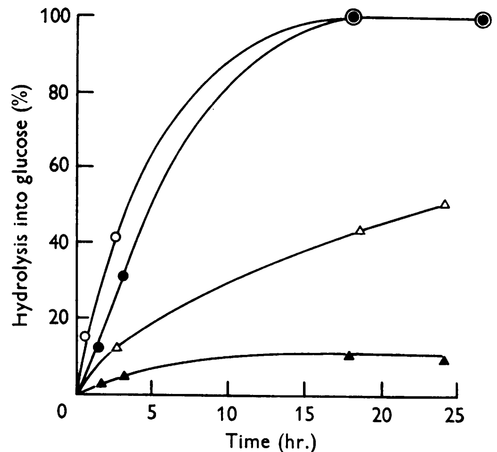

Fig. 9. Comparative action of extracts of *Dasytricha* and *Isotricha* on cellobiose and on maltose. ○, Extract from *Dasytricha* cells (3·4 mg. dry wt.) acting on cellobiose; ●, a similar extract acting on maltose; △, extract from *Isotricha* cells (7·1 mg. dry wt.) acting on cellobiose; ▲, a similar extract acting on maltose. Digests (6 ml.) contained 0·2 M-citrate buffer, pH 5·5, (1 ml.) and were incubated at 35°.

would selectively inactivate the amylase. Mercuric chloride was successfully used by Bailey, Thomas & Whelan (1951), who chose a concentration of $1{\cdot}54 \times 10^{-8}$ moles/unit of phosphorylase to inactivate the small amount of α-amylase which is associated with potato phosphorylase. It was not found possible completely to inactivate holotrich α-amylase by this method unless a concentration of mercuric chloride known to inactivate phosphorylase was chosen.

Wohl & Glimm (1910) reported that α-amylase is inhibited by 15 % glucose. The use of this method for inactivating holotrich amylase was tested as follows. Various amounts of salivary α-amylase were added to potato phosphorylase in standard activity digests containing glucose 1-phosphate and starch 'primer'. After the amount of amylase required to inhibit phosphorylase had been determined, the effect of adding 15 % glucose to the digests was tested. Synthesis, as measured by the liberation of inorganic phosphate, occurred in the tube containing phosphorylase only. No phosphate was liberated in the presence of α-amylase, irrespective of the presence of glucose, and furthermore glucose itself almost completely inhibited phosphorylase action. A similar test carried out with maltose gave the same result. In digests containing 15 % of maltotriose, however, no inhibition of phosphorylase was caused either by the maltotriose or by the α-amylase.

Finally, a portion of freeze-dried holotrich extract (20 mg., 1 ml.) was incubated with glucose 1-phosphate (0·1 M) and maltotriose (500 mg.). Portions (0·3 ml.) were tested for free phosphate by Allen's method (1940). Equilibrium between glucose 1-phosphate and inorganic phosphate was reached in 3 hr. at 35°.

The presence of a phosphatase in the holotrich extract caused some liberation of inorganic phosphate in control digests containing no maltotriose, and this was prevented by the addition of 10^{-4} M-ammonium molybdate. Molybdate also inhibited the phosphoglucomutase action of holotrich extract.

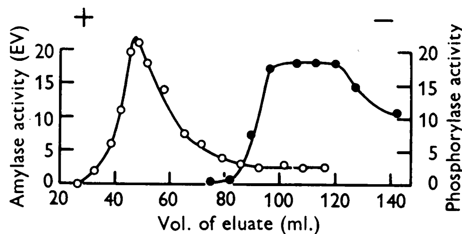

Fig. 10. Distribution of enzyme activity in column-electrophoresis eluate of *Dasytricha* extract (0·1 M-citrate buffer, pH 6·7). ○, Amylase activity; ●, phosphorylase activity.

Phosphorolytic degradation of amylose

A small amount of glucose 1-phosphate was found when a holotrich extract was allowed to react with amylose and phosphate buffer in the presence of ammonium molybdate and mercuric chloride. The percentage conversion into glucose 1-phosphate was very low (2·2 %), since phosphorylase cannot degrade maltose and maltotriose, which are the main products of the amylolytic degradation of amylose.

Electrophoretic separation of phosphorylase and amylase

The possibility of separating amylase from phosphorylase in the holotrich extracts by zone electrophoresis on starch columns was investigated. Washed preparations of *Isotricha* were extracted in 0·1 M-citrate buffer (pH 6·7) and freeze-dried. A portion (46 mg.) of this extract was dissolved in water (1·5 ml.), centrifuged and eluted through the starch column at 20 ml./hr. with 0·1 M-citrate buffer (pH 6·7). The amylase distribution was symmetrical, whereas the phosphorylase was slightly retarded and exhibited adsorption tailing.

The distribution of amylase and phosphorylase activity after electrophoresis on the same column is shown in Fig. 10. The freeze-dried extract (334 mg.) was dissolved in water (2 ml.) and dialysed against water with frequent changes for 2 hr. until the salt concentration was reduced to 0·1 M. After centrifuging, the solution was applied to the column and electrophoresis carried out at 18° for 42 hr. under a p.d. of 258 V and column current 40 mA. The amylase peak was well-defined and separated from the phosphorylase activity, which was widely spread out. A preparation of potato phosphorylase, free from amylase, when subjected to zone electrophoresis under the same conditions gave a sharply defined narrow peak.

Conversion of amylose into branched polysaccharide

Results already presented have shown that the storage polysaccharide of the holotrich Protozoa is amylopectin, and there is no evidence for the presence of any linear polysaccharide. The holotrichs must therefore have a branching enzyme similar to the Q-enzyme of potato, which converts all the linear polysaccharide synthesized by phosphorylase into amylopectin. The test for this branching enzyme was complicated by the presence of amylase, which interfered by hydrolysing both amylose and amylopectin. Amylose was incubated with holotrich extract until the digest became achroic, then a qualitative test was made for isomaltose. It would be expected if the extract contained a branching factor that the amylopectin formed would be broken down by amylase to give

glucose, maltose and isomaltose. Glucose was removed by passing the digest through a charcoal–Celite column, and the disaccharides were eluted with aqueous 25 % (v/v) ethanol, and concentrated to dryness. Further separation of the disaccharides was achieved by zone electrophoresis on paper in borate buffer (pH 8·7), in an apparatus similar to that described by Latner (1952). A p.d. of 400 v was applied overnight, during which time the current rose from 9·5 to 11·5 mA. The paper was coloured with benzidine–trichloroacetic acid reagent (Bacon & Edelman, 1951), and the disaccharide fraction was found to contain both maltose and isomaltose. Isomaltose showed a characteristic pink colour after spraying with benzidine. A mixture of the disaccharides with isomaltose ran as maltose and isomaltose only. The distances moved (cm.) were: maltose 6·5, isomaltose 15·5, glucose 25·5. The same results were obtained with a synthetic amylose, having a chain-length of 55 glucose units, used as substrate in the original digest.

DISCUSSION

The work described in this paper has confirmed the results of Forsyth & Hirst (1953), who identified the storage polysaccharide of the holotrich ciliates as amylopectin. This has been achieved by the use of R-enzyme, which has the property of hydrolysing α-1:6-glucosidic linkages, thus allowing an examination of the inner chains of branched molecules to be carried out. When this enzyme is allowed to act on waxy-maize amylopectin, the intensity of the iodine stain increases to 165 % of its initial value, and the increase is accompanied by a slight shift of the absorption peak towards the red. At the same time the β-amylolysis limit of the amylopectin is raised from 51·5 to 100 %. On the other hand, glycogen treated with R-enzyme is not hydrolysed to any greater extent than glycogen by either α- or β-amylase, nor does the presence of active R-enzyme influence the degree of hydrolysis by crystalline β-amylase. The glycogen molecule is much more compact than amylopectin, the basal chains being shorter and the branch linkages nearer together, and it has been suggested (Peat *et al.* 1956) that this high degree of ramification renders the branch linkages of glycogen inaccessible to R-enzyme. A means of separating these linkages is provided by α-amylolytic degradation of glycogen, and this treatment does in fact render the branch linkages susceptible to R-enzyme action. The results for holotrich starch have shown that R-enzyme can increase the iodine absorption value of this starch, and the resulting starch treated with R-enzyme is completely hydrolysed to maltose by α-amylase. Further, the β-amylolysis limit of 51·5 % conversion into maltose is raised to 101 % when R-enzyme and β-amylase act simultaneously on the starch. This proves that holotrich starch is a typical amylopectin.

The results have also confirmed that the starch may be synthesized by the phosphorolytic mechanism common to both the plant and animal kingdoms. Other workers (Masson & Oxford, 1951) have determined which sugars may be utilized by the holotrichs for starch synthesis *in vivo*. The enzymes required for the hydrolysis of these carbohydrates to give glucose and fructose have all been detected in holotrich extract, and their activity estimated. Sugden & Oxford (1952) have reported that glucose 1-phosphate, when added to holotrich Protozoa in a suitable buffer, did not prolong their life, whereas the addition of cellobiose, fructose, glucose, sucrose, raffinose, inulin or bacterial levan extended the life of the culture. It is likely that glucose 1-phosphate is barred from permeating through the cell wall while the free sugars enter the cell, where they are hydrolysed to glucose and fructose by the appropriate enzyme and are then phosphorylated by hexokinase.

Maltose is the only disaccharide which can be hydrolysed to glucose without subsequent storage of starch. It is true that the maltase activity determined in holotrich extract is of a low order, yet it is comparable with cellobiase activity, and the holotrichs can utilize cellobiose for storage of starch. An explanation might be that maltose is utilized preferentially by some other enzyme system so far undetected.

Table 3. *Summary of properties of α-amylases*

The table is taken from Fischer & de Montmollin (1951*a*, *b*), with the addition of data for A_D and A_I obtained in the present work. Abbreviations: G, glucose; M, maltose; T, maltotriose.

	Malt	*Aspergillus oryzae*	A_D	A_I	Rumen *Streptococcus bovis*	*Bacillus subtilis*	Pig pancreas	Human saliva
Optimum pH	4·1, 5·4	5·5–5·9	5·0, 6·0	4·8	5·5–6·5	5·3–6·8	6·9	6·9
Activation								
By Ca^{2+} ions	+	–	–	–	–	–	–	–
By Cl^- ions	–	–	–	–	–	+	+	+
Isoelectric point	5·6	4·2	<5	5·4	.	.	5·3	5·3
Product of action on amylose	G, M	G, M	G, M, T	M, T	M, T′	G, M	G, M	G, M, T

The zone-electrophoretic fractionation of aqueous extracts of holotrichs has overcome some of the difficulties in a complete physical separation of the *Isotricha* and *Dasytricha* and has permitted the characterization of the α-amylases specific to each organism. The overlapping of the two activity curves in varying proportions would otherwise have obliterated the characteristic pH optima given by the two enzymes.

It is of interest to compare the properties of the holotrich amylases with those of other amylases of plant and animal origin. For this purpose our results have been combined with those of Fischer & de Montmollin (1951*a*, *b*) in Table 3. A_D and A_I may be grouped with the amylases of *Aspergillus oryzae* and the rumen *Streptococcus* (Hobson & Macpherson, 1952), all of which are independent of the presence of Cl^- or Ca^{2+} ions.

An attempt has been made to fractionate these enzymes by absorption chromatography on hydroxyapatite columns eluted with phosphate buffer of increasing concentration (Tiselius, Hjertén & Levin, 1956; Semenza, 1957). Differences in the elution curves and in the activity–pH curves of the eluted peaks of single organism preparations are evident although the enzymes have not been completely resolved. The zone-electrophoretic fractionations so far appear to have been more satisfactory.

SUMMARY

1. The action of the debranching enzyme R-enzyme, α-amylase and β-amylase on starch stored by holotrich ciliates from the sheep rumen has verified that this starch is amylopectin.

2. A comparison of various enzyme activities present in aqueous extracts of purified preparations of the single organisms *Isotricha* and *Dasytricha* has been made.

3. In the presence of 15% maltotriose, holotrich α-amylase is inhibited but phosphorylase activity is unaffected.

4. Holotrich starch is synthesized by the phosphorolytic mechanism common to plants and animals.

5. Two α-amylases specific to *Isotricha* and *Dasytricha* have been resolved by zone electrophoresis of holotrich extracts on starch columns.

6. These amylases differ in optimum pH, electrophoretic mobility and isoelectric points. They are not activated by Ca^{2+} or Cl^- ions.

7. Zone-electrophoretic separation of phosphorylase and amylase was unsuccessful owing to adsorption of phosphorylase on the column.

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