Studies on Carbohydrate-Metabolizing Enzymes

THE HYDROLYSIS OF α-GLUCOSIDES, INCLUDING NIGEROSE, BY EXTRACTS OF ALFALFA AND OTHER HIGHER PLANTS

By D. H. HUTSON and D. J. MANNERS

Department of Chemistry, University of Edinburgh

1. Enzyme preparations from 11 plant sources, from yeast and from the protozoan *Tetrahymena pyriformis* show nigerase activity, which, in most preparations, was 70–90% of that towards maltose. 2. These enzyme preparations also hydrolysed isomaltose, but there was a wide variation in relative maltase to isomaltase activity. 3. The maltase and nigerase activities of alfalfa and tomato preparations could not be differentiated by heat inactivation or inhibitor methods. However, with turanose used as a competitive inhibitor, evidence suggesting that maltose and nigerose are hydrolysed at different catalytically active sites in the alfalfa preparation was obtained. 4. It is probable that the alfalfa α-glucosidase exists as a mixture of isoenzymes.

Enzymes which hydrolyse α-glucosidic linkages are widely distributed in Nature; for example, extracts of various land plants, seaweeds, protozoa, fungi, bacteria, vertebrates and invertebrates contain maltase (for a review see Larner, 1960). During a survey of the carbohydrase activity of alfalfa (lucerne) extracts (Hutson & Manners, 1964), strong maltase activity was observed; however, the degradation of soluble nigeran [a linear polymer of D-glucopyranose containing alternate α-(1→4)- and α-(1→3)-glucosidic linkages which is resistant to α- and β-amylolysis] to glucose by the extracts was an unexpected feature. This breakdown indicated that one or more α-(1→3)-glucosidases were present in the extracts. Subsequent tests showed that, as expected, nigerose was readily hydrolysed, but at a slightly lower rate than maltose. Although nigerase activity has previously been observed in animal, fungal and bacterial preparations (e.g. Larner & Gillespie, 1956; Pazur & Kleppe, 1962; Bailey & Robertson, 1962), the only report of activity in the plant kingdom appears to be that of Peat & Rees (1961), who detected nigerase in extracts of *Porphyra umbilicalis*. Although the absence of nigerase activity from extracts of certain bacteria (Bailey & Bourne, 1961) and yeast (Phillips, 1959) has been reported, it is apparent that the scarcity of the substrate is a major reason for the present lack of knowledge on the distribution of α-(1→3)-glucosidases. Accordingly, we have surveyed a number of plant extracts for this activity; a preliminary account of this survey has been published (Hutson & Manners, 1963).

In general, plant α-glucosidases differ from β-glucosidases in that they show a much higher degree of specificity (cf. Larner, 1960). Fractionation studies of yeast α-glucosidase, and the finding of fluctuations in the ratios of certain activities, e.g. maltase and trehalase (Hestrin & Lindegren, 1952; Winge & Roberts, 1950), maltase and methyl α-D-glucosidase (Hestrin & Lindegren, 1952; Robertson & Halvorson, 1957), maltase and sucrase (Sawai, 1956), have led to suggestions of the existence of various α-glucosidases with restricted substrate specificities. These studies, however, have not included nigerase activity.

The presence of nigerase in plant extracts is of particular interest since the α-(1→3)-glucosidic linkage occurs only rarely in plants (e.g. isolichenin in Iceland moss). An attempt has therefore been made to determine whether the nigerase activity of alfalfa is due to a group-specific α-glucosidase, the main function of which is the hydrolysis of maltose, or is due to the presence of a specific α-(1→3)-glucosidase.

MATERIALS

Plant tissue. Untreated alfalfa seeds (var. de Puits) were purchased from Elsom Ltd., Spalding, Lincs., and allowed to germinate in a moist, dark box at 26° for 4 days to produce seedlings. The other plant tissues were fresh seedlings, or as described in Table 3.

Carbohydrates. Maltose, obtained commercially, was twice recrystallized from methanol and contained less than 0·5% of glucose. Nigerose was prepared by the acetolysis of a branched dextran (synthesized from sucrose by *Leuconostoc mesenteroides*, NCIB 2706) by the method of Matsuda, Watanabe & Aso (1961). Nigeran pentasaccharide alcohol

was prepared by the sodium borohydride reduction of the tetraose–pentaose mixture of a partial hydrolysate of nigeran which was a gift from Dr H. Weigel. Maltitol was prepared by the sodium borohydride reduction of maltose. Kojibiose was a gift from Dr K. Matsuda.

METHODS

Preparation of alfalfa seedling extract. Four-day-old alfalfa seedlings (450 g. fresh wt.) in 0·4 M-sodium acetate (2 l., pH 7·5) at 0° were treated for 20 min. with an Ultra-Turrax TP18 homogenizer, the temperature being kept below 10°. The mixture was stirred for 1 hr. at 18°, adjusted to pH 6 with acetic acid solution and filtered through muslin. The centrifuged extract was dialysed overnight against tap water (8°) and then clarified by treatment in a Sharples centrifuge to give 3 l. of protein solution. Ammonium sulphate (1560 g.) was added to the stirred solution cooled with ice and the solution stored overnight at 0°. The protein was collected by centrifugation, dissolved in water, dialysed, centrifuged, made 0·01 M with sodium citrate (pH 6·0) and freeze-dried. The protein content of the resulting preparation, as measured by a quantitative biuret method (Robinson & Hogden, 1940), was 52%.

Glucose assay. Enzyme action was assayed by determination of the liberated glucose with the glucose oxidase reagent described by White & Subers (1961). Control experiments showed that the reagent, which contained tris buffer to inhibit contaminating glycosidases, had no action on maltose, nigerose or isomaltose.

Reducing power. This was determined with 3,5-dinitrosalicylic acid (Sumner, 1924) standardized against glucose.

General incubation conditions. Standard digests contained sugar (2·5 mg./ml.; 2 vol.), McIlvaine buffer (1 vol.) and plant extract (1 vol.) and were incubated at 37°. Appropriate controls were prepared for each experiment.

α-Glucosidase activity. This was assayed by incubating 2 or 4 ml. digests of the above composition for 17–24 hr., inactivating the enzyme by heating at 100° for 3 min., and determining the concentration of glucose in 2 ml. portions of suitably diluted solutions.

Heat-treatment experiments. These were carried out by immersing enzyme–buffer mixtures (1:1, v/v) in a water bath at the required temperature for a fixed time and then immediately cooling in ice–water. For experiments on the rate of inactivation, the enzyme–buffer mixture was preheated to the required temperature before the initial sample was withdrawn. Enzyme solutions were stored at 0° until assayed for activity.

Continuous electrophoresis. The instrument used was the Beckman–Spinco model CP. The separation of alfalfa extract was carried out in McIlvaine buffer, diluted ten times with water (final pH 6·6). The voltage was 580 v and the current 85 mA: Schleicher and Schüll no. 470 filter paper was used, cut to provide 32 drip-points at the bottom of the curtain.

The paper-chromatographic methods used are described by Hutson & Manners (1964).

RESULTS

Specificity of seedling α-glucosidase. The pH optima of the maltase and nigerase activities were previously found to be in the range 4·5–5·0 (Hutson

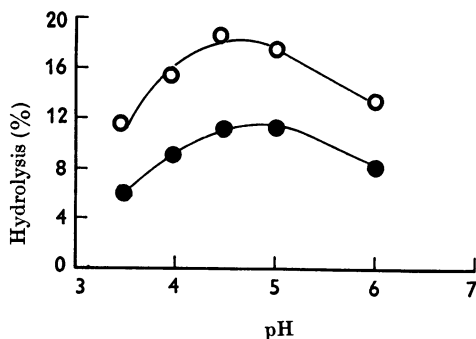
Fig. 1. Effect of pH on the hydrolysis of maltose (○) and nigerose (●) by alfalfa seedling extract.

Table 1. *Rates of hydrolysis of various α-glucosides by alfalfa seedling extract at pH 4·6*

Substrate	Hydrolysis (%/hr./mg. of protein)	Activity relative to maltase
Maltose	1·33	100
Nigerose	1·00	75
Isomaltose	0·34	26
Kojibiose	0·32	24
Trehalose	1·04	78
Maltitol	0·12	9
Turanose	0·01	1
Sucrose	1·61	121
Methyl α-D-glucoside	0·01	1

& Manners, 1964), and this has been confirmed in a reinvestigation of the two activities (Fig. 1).

The initial work was carried out at pH 4·6–4·8 with the seedling extract described in the Methods section. The maltase activity of this extract, which contained 52% of protein, freeze-dried in sodium citrate, was stable over several months when stored at 0°. A solution of the extract (1–2 mg./ml.) was incubated under standard conditions with several α-glucosides at final concentrations of 1·25 mg./ml. (3·66 mM). After assaying with glucose oxidase, the specific activity of the extract towards the various substrates was calculated. These results are shown in Table 1, together with activity figures relative to the maltase activity. When enzyme solutions of 1–2 mg./ml. were used for incubation, the concentration of glucose in the control digests was negligible. From 0 to 26% hydrolysis of nigerose, there was a linear relationship between the enzymic activity and the concentration of extract (0·8–3·6 mg./4 ml. of digest), and in the various experiments the concentrations of extract were within this range.

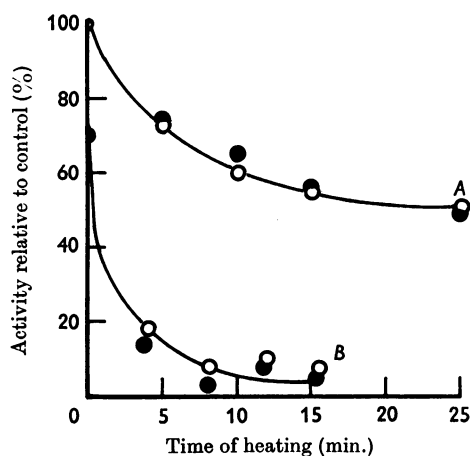The ratio maltase:nigerase (about 100:75) was

Fig. 2. Effect of heating at 60° (*A*) and 70° (*B*) on the maltase (○) and nigerase (●) activity of alfalfa seedling preparation.

Table 2. *Effect of various inhibitors on alfalfa seedling maltase and nigerase activity*

Experimental details are given in the text.

Inhibitor	Concn. (mM)	Maltase	Nigerase
Mercuric chloride	0·01	2	7
	1·0	80	80
Tris	100	55	52
	200	60	60
Glucono-(1→5)-lactone	1·0	4	0
	4·0	11	18
	10·0	28	34
Turanose	3·66	34	52
	7·32	41	72

subject to a small variation over many determinations but the general range was 100:73–77.

Action of the seedling extract on reduced nigeran pentasaccharide. A standard digest containing twice the usual concentration of substrate was incubated with enzyme solution (2mg./ml.). The yield of glucose after 0, 5, 24 and 70hr. was 0, 2·4, 8·2 and 22%. Part of the digest, sampled at 24hr., possessed a reducing power equivalent to 7·2% hydrolysis. Thus the sole reducing sugar is glucose. This was confirmed by chromatography of a similar digest, glucose being the only detectable reducing sugar.

Heat-inactivation of maltase and nigerase. Two types of experiment were carried out. In the first series, seedling preparation (2mg./ml.) was mixed with an equal volume of McIlvaine buffer (final pH 4·5) and portions were heated for 10min. at various temperatures. Maltase and nigerase activities were determined and calculated as percentages of the activities in a freshly prepared, untreated enzyme–buffer mixture. After heating at 20°, 40°, 50° and 65°, the maltase activity was 100, 100, 99 and 53 respectively, and that towards nigerose was 100, 97, 90 and 51.

In the second experiment, the rate of inactivation of the enzymes at constant temperature was measured. Temperatures of 60° and 70° were chosen and enzyme–buffer mixtures (pH 4·6) were treated as described in the Methods section. As shown in Fig. 2, both activities were inactivated more rapidly at 70° than at 60°, values after 15min. being about 10 and 55% respectively of the original, but at neither temperature could the activities be differentiated.

Inhibition experiments

Mercuric chloride. The effect of Hg^{2+} ions on the seedling maltase and nigerase was studied by incubation under standard conditions in the presence of 1·0 and 0·01mM-mercuric chloride. The results are expressed, relative to a control digest, in Table 2. Control experiments showed that 1·0mM-mercuric chloride did not interfere with the glucose oxidase reagent.

2-Amino-2-hydroxymethylpropane-1,3-diol. Tris buffer is known to inhibit maltase (Dahlqvist, 1961) and is used as a component of glucose oxidase–peroxidase reagents to suppress maltase activity in crude glucose oxidase preparations. Standard digests containing tris buffer (0·1 and 0·2M) were investigated. The results are shown in Table 2.

Glucono-(1→5)-*lactone.* The inhibitory action of glucono-(1→5)-lactone (cf. Conchie & Levvy, 1957) was examined. The digests were of standard composition but freshly prepared inhibitor was added to give final concentrations of 1, 4 and 10mM. Since the lactone slowly hydrolyses, the concentration of inhibiting species did not remain constant; nevertheless, a satisfactory comparison of the two activities could be made. Table 2 shows the degree of inhibition of the activities.

Turanose. In the presence of turanose (3-*O*-α-D-glucopyranosyl-D-fructose), competitive inhibition of both maltase and nigerase was observed. Since the concentration of inhibitor remained constant, it was considered to be more satisfactory than glucono-(1→5)-lactone for use under the present conditions. Standard digests were prepared with appropriate controls, and assayed with the glucose oxidase reagent. Inhibitor concentrations which, with respect to substrate concentration, were (*a*) equimolar and (*b*) twice this value, were used. The effect of this inhibitor is shown in Table 2.

Ammonium sulphate fractionation of seedling extract

A crude seedling extract containing about 8 mg. of protein/ml. was fractionated, by the addition of solid ammonium sulphate, into three fractions: 0–30%, 30–40% and 40–70% saturation. The fractions were dialysed and freeze-dried in sodium citrate. The last fraction (no. 3) contained excessive amounts of salt, and was dissolved in water, dialysed and again freeze-dried. The ratio of nigerase to maltase activity was virtually unchanged during the fractionation (0·77, 0·73 and 0·74 respectively in the successive fractions) and the relative specific activity of the maltase increased (1·0:2·3:3·8) with increasing concentration of ammonium sulphate.

Continuous electrophoresis

The seedling extract was subjected to continuous electrophoresis at pH 6·6 as described in the Methods section. Fraction no. 3, which possessed a high specific activity and was more soluble than other preparations, was used at a concentration of 20 mg./ml. A total of 118 mg. of protein was applied to the curtain at a point vertically above tube 7. The distribution of protein amongst one rack of 32 tubes (from extinction measurements at 280 mμ) and maltase activity (based on the glucose oxidase method of assay) are shown in Fig. 3.

The three peaks of maltase activity [A (tubes 5–9), B (tubes 10–12) and C (tubes 13–20)] were collected, made 0·01 M with sodium citrate (pH 6·0) and stored at 0°. Assay of maltase activity revealed that A contained 53%, B, 7% and C, 32% of the original activity, representing a total recovery of 92%.

Concentration of fraction A by using dry Sephadex G-25 (Flodin, Gelotte & Porath, 1960) followed by an ultrafiltration (LKB, Uppsala, apparatus),

led to a 70% loss of activity. Fractions B and C were concentrated by freeze-drying and dialysed to remove excess of salt.

Stability and specificity of fractions A, B and C

Stability. Fractions A, B and C were assayed for maltase activity after being stored under the following conditions: (a) at 2° (control), (b) at 20° for 4 days, (c) at 37° for 20 hr., (d) at 50° for 10 min. For treatments (b)–(d) the fractions were diluted with an equal volume of buffer, pH 4·6; for treatments (b) and (c) the solutions were stored under toluene. The maltase activities, expressed as percentages of the control (which showed no change during 7 days), were: fraction A, (b) 78, (c) 86, (d) 80; fraction B, (c) 87, (d) 79; fraction C, (b) 100, (c) 100, (d) 90. It is clear that fractions A and B are more labile than fraction C.

Specificity. The maltase, nigerase and isomaltase activities in fractions A, B and C were determined by standard methods, and the following results (expressed as a percentage of the maltase activity) were obtained: nigerase:isomaltase, fraction A, 78:26, fraction B, 40:66, fraction C, 77:44. The results show a significant variation in the activity of the three alfalfa fractions.

Further studies on fraction C

Optimum pH. The optimum for both maltase and nigerase activity was about pH 4·6.

Michaelis constants. Maltose and nigerose were incubated at various concentrations with fraction C under the following conditions: substrate (1·25–10 mg./ml.; 8 ml.), McIlvaine buffer (pH 4·5, 4 ml.), enzyme (fraction C solution, 4 ml.). The final pH of the digests was 4·6. The concentrations of substrates in the digests were 1·825, 3·65, 7·3 and 14·6 mM. The digests were incubated at 37° and assayed at appropriate time-intervals to obtain an accurate measure of the initial rate of hydrolysis. The rate of liberation of glucose was linear with time in all digests, hence v, the initial velocity of hydrolysis, could be readily estimated. A typical Michaelis–Menten relationship was observed when $1/S$ was plotted against $1/v$ (Lineweaver & Burk, 1934). The K_m values calculated from this graph were 0·83 mM for maltose and 2·30 mM for nigerose. Under similar conditions, fraction A had K_m 1·35 mM for maltose.

Inhibition by turanose. In the presence of turanose (incorporated into the buffer at a concentration of 10 mg./ml., giving a final concentration of 7·3 mM) the slope of the plot of $1/v$ against $1/S$ increased, while the intercept with the ordinate remained constant (Fig. 4), thus indicating competitive inhibition (Lineweaver & Burk, 1934). The
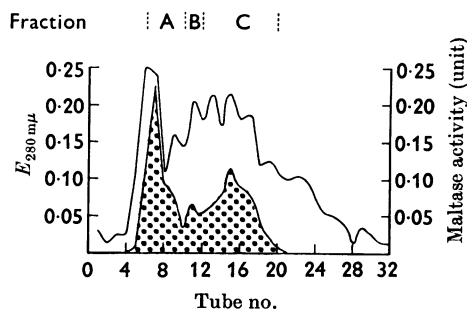

Fig. 3. Distribution of protein and maltase activity (shaded area) during continuous electrophoresis of alfalfa seedling preparation.

inhibitor constant (K_i) for turanose in the hydrolysis of maltose was 5·56mM and for the hydrolysis of nigerose 2·18mM, suggesting that the activities did not take place at the same catalytic site.

Hydrolysis of nigerose by extracts of other plants

Several plant tissues were extracted as described in the Methods section and the extracts tested for nigerase activity. The various sources, yields of solu-ble protein and nigerase activities are listed in Table 3. The maltase and isomaltase activities of certain of these extracts, determined under similar conditions, are also included. A comparison of the tomato maltase and nigerase by heat-denaturation, and degree of inhibition by Hg^{2+} ions, turanose or gluconolactone, gave results very similar to those of the alfalfa extract. Heat and Hg^{2+} ions affected the two activities similarly and the competitive inhibitors affected the nigerase activity more than the maltase. For example, heat treatment for 10min. at 45°, 55° and 65° reduced the maltase activity by 5, 16 and 96%, and the nigerase activity by 0, 14 and 98%. The results of the inhibition experiments are shown in Table 4.

Other enzyme preparations

The relative nigerase and isomaltase activities of a commercial yeast maltase preparation (L. Light and Co. Ltd.), and a freeze-dried extract of the protozoan *Tetrahymena pyriformis* (cf. Archibald & Manners, 1959), were measured, as in Table 3, and
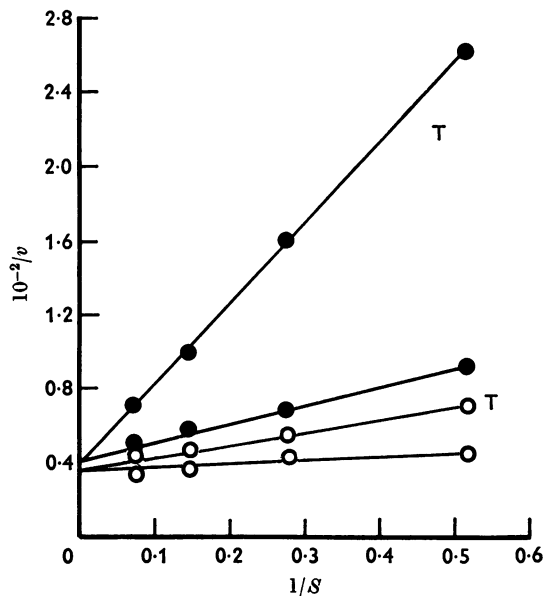

Fig. 4. Effect of turanose (T) on the hydrolysis of maltose (○) and nigerose (●) by fraction C. Substrate concentration (S) is expressed as mM, and initial velocity (v) as mg. of disaccharide hydrolysed/2 ml. of digest/hr.

Table 4. *Effect of various inhibitors on the maltase and nigerase activity of the tomato preparation*

Experimental conditions were those used in Table 2.

Inhibitor	Concn. (mM)	Percentage inhibition	
		Maltase	Nigerase
Mercuric chloride	1·0	90	91
Glucono-(1 → 5)-lactone	1·0	3	2
	4·0	4	10
	10·0	17	33
Turanose	3·66	60	68
	7·32	71	76

Table 3. *Extraction of protein from various plant tissues and estimation of the resultant α-glucosidase activities*

Plant source	Yield of protein* (%)	Hydrolysis by α-glucosidase (%/hr./mg. of protein)			Nigerase:isomaltase (expressed as percentages of maltase values)
		Nigerase	Maltase	Isomaltase	
Tomato (fruit)	0·04	4·03	4·15	0·29	92:7
Lupin (leaves and stems)	0·12	3·50	—	—	—
Carrot (root)	0·03	3·16	4·43	2·21	71:50
Alfalfa (seedling)	1·00	1·15	1·49	0·39	71:26
Malted barley	0·57	1·07	1·23	0·21	87:17
Pea (seedling)	1·07	0·62	—	—	—
Soya bean (seedling)	1·53	0·60	0·80	0	75:0
Barley (seed)	0·57	0·42	0·40	0·04	106:10
Potato (tuber)	0·23	0·39	0·46	0	85:0
Runner bean (seedling)	1·23	0·26	—	—	—
Sweet almond (seed)	2·41	0·08	0·28	0·04	29:17

* Based on weight of fresh tissue except for seedlings (based on seed weight).

found to be 85:16 and 73:39 respectively (expressed as a percentage of the maltase activity).

DISCUSSION

The present result ssuggest that α-(1→3)-glucosidase activity is widely distributed in Nature, since nigerase activity is shown by extracts of 11 plant sources, of *Tetrahymena pyriformis*, by a yeast maltase preparation (although this last observation contrasts with that of Phillips, 1959), and by the animal and microbial preparations cited in the introduction to this paper. The nigerase is closely associated with maltase and isomaltase activities, but, with the exception of sweet almonds, the maltase:nigerase ratio for the various plant extracts is similar to the value 100:75 found by Lukomskaya (1962) for a rabbit-liver preparation. By contrast, there is a marked variation in the ratio of maltase:isomaltase activities in the various plant preparations (Table 3). Since the α-(1→3)-glucosidic linkage occurs only rarely in Nature (e.g. in nigeran, isolichenin and as interchain linkages in certain dextrans), it seems probable that the nigerase activity has little or no metabolic importance, and represents one catalytic feature of a general α-glucosidase. The degradation of the reduced pentasaccharide from nigeran to glucose is probably the result of endwise α-glucosidase action rather than that of a true polysaccharase.

In view of the presence of a significant amount of maltase activity in the sweet-almond extract, the frequent use of almond emulsin as a source of β-glucosidase for the determination of the configuration of glucosidic linkages in oligosaccharides requires caution.

The alfalfa-seedling preparation has been studied in some detail. The original extract hydrolysed each of the five α-linked glucose disaccharides, but at very different rates; maltitol was only slowly attacked, and methyl α-D-glucoside and turanose were not measurably hydrolysed (less than 1%). The maltase activity was not inhibited by methyl α-D-glucoside, but turanose was a competitive inhibitor of both the maltase and nigerase activities. The extract readily hydrolysed sucrose, but as this activity was optimum at pH 6·0, whereas that towards maltose and nigerose was optimum at pH 4·6, a different enzyme (presumably a β-fructofuranosidase) is involved. This view is supported by the fact that the invertase activity is not inhibited by either turanose or glucono-(1→5)-lactone (unpublished results).

The hydrolytic activity of the seedling extracts is due to plant enzymes, and not to contaminating bacterial enzymes, since control experiments have shown that the activity of extracts of alfalfa seeds germinated under sterile conditions was identical with that of seeds germinated under the usual conditions (Hutson & Manners, 1964).

The relationship between the alfalfa maltase and nigerase activities was investigated by ammonium sulphate fractionation of an extract, by attempted selective inactivation on heating and selective inhibition with mercuric chloride and tris; all these experiments failed to distinguish between the two activities. With competitive inhibitors [turanose and glucono-(1→5)-lactone] the nigerase activity was reduced more than the maltase, but this fact also does not differentiate between the activities, since an enzyme with a higher affinity for maltose than for nigerose will show a smaller decrease in maltase activity for a given competitive inhibitor than in nigerase activity.

An attempt was made to separate the maltase and nigerase activities by continuous electrophoresis. This was not successful, although it was possible to separate the enzymically active protein into three fractions, each of which showed varying ratios of maltase, nigerase and isomaltase activities. This suggests that in alfalfa α-glucosidases exist as isoenzymes, a term originally applied to electrophoretically different forms of a protein which showed the same enzymic specificity (Markert & Moller, 1959), but more recently used to describe a family of enzymes having the same general specificity but differing in the details of their catalytic and physical properties. The existence of carbohydrase isoenzymes in the animal kingdom is evident from the studies of Dahlqvist (1960a) on hog intestinal α-glucosidases. The electrophoretic fractions A and B were too unstable or obtained in too low a yield to permit detailed kinetic studies, so that the following results refer only to fraction C.

This fraction showed the normal ratio of maltase:nigerase activity, and the ratio maltase:isomaltase was intermediate between that in fractions B and in fraction A. K_m for maltose was 0·83mM, which is intermediate between the K_m values for a purified hog intestinal maltase or Taka-maltase (4·2 or 3·5mM respectively; Dahlqvist 1960b; Kato, Matsushima & Akabori, 1960) and that of yeast maltase (0·28mM; Hestrin, 1961). Unlike a hog intestinal preparation, which showed the same K_m values for maltose and nigerose (Larner & Gillespie, 1956), fraction C had K_m 2·30mM for nigerose. A further difference from the animal enzyme system is that, although turanose is a competitive inhibitor, it is not a substrate for the alfalfa preparation (cf. Dahlqvist, 1959).

The presence of isomaltase in malted barley (var. Herta) was reported recently by Jørgensen (1963), although the ratio of isomaltase:maltase activity was only about 1:16, whereas with our sample of malted barley (var. Ymer) this ratio was

about $1:6$, and with ungerminated barley about $1:10$.

In general, the results in Table 3 indicate that the α-glucosidases of higher plants are not highly specific 'maltases', but show activity towards α-$(1\rightarrow3)$-, α-$(1\rightarrow4)$- and α-$(1\rightarrow6)$-glucosidic linkages. In alfalfa and tomato preparations, the first two activities cannot be separated by simple fractionation or inactivation methods. Competitive-inhibitor studies (Fig. 4) suggest that with the alfalfa preparation, maltose and nigerose are hydrolysed at different catalytically active sites. Whether this is due to different enzyme proteins, and whether, as in malted barley (cf. Jørgensen, 1963), isomaltose and maltose are hydrolysed at the same reactive site, is being further investigated.

REFERENCES

Archibald, A. R. & Manners, D. J. (1959). *Biochem. J.* **73**, 292.

Bailey, R. W. & Bourne, E. J. (1961). *Nature, Lond.*, **191**, 277.

Bailey, R. W. & Robertson, A. M. (1962). *Biochem. J.* **82**, 272.

Conchie, J. & Levvy, G. A. (1957). *Biochem. J.* **65**, 389.

Dahlqvist, A. (1959). *Acta chem. scand.* **13**, 2156.

Dahlqvist, A. (1960a). Dissertation: University of Lund.

Dahlqvist, A. (1960b). *Acta chem. scand.* **14**, 1.

Dahlqvist, A. (1961). *Biochem. J.* **80**, 547.

Flodin, P., Gelotte, B. & Porath, J. (1960). *Nature, Lond.*, **188**, 493.

Hestrin, S. (1961). In *Biochemist's Handbook*, p. 221. Ed. by Long, C. London: E. and F.N. Spon Ltd.

Hestrin, S. & Lindegren, C. (1952). *Arch. Biochem. Biophys.* **38**, 317.

Hutson, D. H. & Manners, D. J. (1963). *Biochem. J.* **89**, 91P.

Hutson, D. H. & Manners, D. J. (1964). *Biochem. J.* **93**, 545.

Jørgensen, O. B. (1963). *Acta chem. scand.* **17**, 2471.

Kato, I., Matsushima, T. & Akabori, S. (1960). *J. Biochem., Tokyo*, **48**, 199.

Larner, J. (1960). In *The Enzymes*, vol. 4, part A, p. 369, Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.

Larner, J. & Gillespie, R. E. (1956). *J. Amer. chem. Soc.* **78**, 882.

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

Lukomskaya, I. S. (1962). *Biokhimiya*, **27**, 875.

Markert, C. L. & Moller, F. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 753.

Matsuda, K., Watanabe, H. & Aso, K. (1961). *Tohoku J. agric. Res.* **12**, 351.

Pazur, J. H. & Kleppe, K. (1962). *J. biol. Chem.* **237**, 1002.

Peat, S. & Rees, D. A. (1961). *Biochem. J.* **79**, 7.

Phillips, A. W. (1959). *Arch. Biochem. Biophys.* **80**, 346.

Robertson, J. J. & Halvorson, H. O. (1957). *J. Bact.* **73**, 186.

Robinson, H. W. & Hogden, C. G. (1940). *J. biol. Chem.* **135**, 727.

Sawai, T. (1956). *Bot. Mag. Tokyo*, **65**, 193.

Sumner, J. B. (1924). *J. biol. Chem.* **62**, 287.

White, J. W. & Subers, M. H. (1961). *Analyt. Biochem.* **2**, 380.

Winge, O. & Roberts, C. (1950). *C.R. Lab. Carlsberg, Sér. physiol.* **25**, 35.