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Studies on Carbohydrate-Metabolizing Enzymes

7. YEAST ISOAMYLASE*

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Although the starch- and glycogen-metabolizing enzymes of higher plants and animals have been intensively studied [for reviews, see Manners (1953) and Whelan (1958)], the related yeast enzymes have attracted comparatively little attention. For example, the presence of phosphorylase in yeast extracts was reported by Schaffner & Specht (1938) and Cori, Colowick & Cori (1938), but the enzyme does not appear to have been highly purified, whereas both potato and muscle phosphorylase have been crystallized. Further, the properties of yeast branching enzyme have only recently been reported (Gunja, Manners & Khin Maung, 1960), although potato Q-enzyme was first investigated 16 years ago.

This paper is concerned with a yeast 'debranching' enzyme, hereafter referred to as isoamylase, which hydrolyses the outer α-(1 $\rightarrow$ 6)-glucosidic inter-chain linkages in amylopectin and glycogen. Nishimura (1931) noted that yeast extracts contained an enzyme, 'amylosynthease', which could liquefy starch and accelerate the action of normal amylases, although it differed in thermolability from α- and β-amylases. Further studies by Nishimura & Minagawa (1931) and Minagawa (1932) showed that enzyme action on glutinous rice starch, which was optimum at pH 6·2 and 20°, caused an increase in iodine-staining power. Amylosynthease was therefore regarded as a starch-synthesizing enzyme. Later, Meyer & Bernfeld (1942) observed that yeast extracts attacked both starch and its β-dextrin. A small increase (7–15%) in the β-amylolysis limit of the latter was noted. The impure enzyme preparation also hydrolysed maltose and isomaltose. The action of amylosynthease on rice starch was re-examined by Maruo & Kobayashi (1951). The product had a lower molecular weight, higher β-amylolysis limit, stained bluish-purple with iodine, and showed a tendency to retrograde from solution. These results were considered to be caused by extensive debranching of the starch, and the new name isoamylase was proposed for the enzyme. This implies a starch-degrading function. This activity is similar to that of R-enzyme on amylopectin (Hobson, Whelan & Peat, 1951).

In view of our interest in the structure and metabolism of glycogen (Manners, 1957), the action of yeast isoamylase on this and related polysaccharides was investigated. It should be noted that R-enzyme has no action on normal glycogen (Peat, Whelan, Hobson & Thomas, 1954; Fleming & Manners, 1958). As stated in the preliminary account of this work (Manners & Khin Maung, 1955) we first encountered isoamylase as an impurity in yeast-phosphorylase preparations.

* Part 6: Liddle, Manners & Wright (1961).

METHODS AND MATERIALS

Analytical methods. The methods used for paper chromatography, the determination of reducing sugars and the analysis of polysaccharides (by α- and β-amylolysis and periodate oxidation) have been described (Gunja *et al.* 1960).

Determination of isoamylase activity. The action of a debranching enzyme causes an increase in both the β-amylolysis limit, and iodine-staining power of glycogen but no appreciable increase in reducing power. The changes may be used as a measure of activity although since neither property is directly related to the number of α-(1$\rightarrow$6)-glucosidic linkages hydrolysed, such values are relative rather than absolute.

Digests containing approximately equal weights of glycogen and isoamylase preparation (usually 1 or 2 mg./ml. in a total volume of 25 ml.) were incubated at pH 5·9

and room temperature (18–20°) for 18 hr. Unless otherwise stated, 0·2M-sodium acetate buffer, pH 5·9 or 6·0, was used. For β-amylolysis, a portion was heated (3 min. at 98°) and then cooled, and β-amylase added; the maltose content was then measured after incubation for 24 hr. at 35°. For iodine-staining, samples (2 or 3 ml.) were stained with iodine solution [0·2% of iodine in 2% (w/v) potassium iodide; 1 or 2 ml.], acidified (3 drops of 5N-hydrochloric acid) and diluted to 25 ml. with water, and the extinction was measured on a Unicam SP. 500 spectrophotometer at a wavelength in the region 425–480 mμ (depending upon the wavelength of maximum absorption of the particular glycogen sample) against an iodine–water blank.

Enzyme preparations. The samples of pressed brewer's yeast (*Saccharomyces cerevisiae*) and α- and β-amylase were those used by Gunja *et al.* (1960).

Substrates. Glycogen and amylopectin samples were as described by Gunja *et al.* (1960). Maltodextrin (a mixture of linear and branched maltosaccharides produced by the action of malt α-amylase on potato starch) was kindly provided by Professor C. S. Hanes, F.R.S.

Nomenclature. In multiply branched molecules of the glycogen–amylopectin type, A-chains are defined as those which are attached to the rest of the molecule by a single linkage from the reducing group, whereas B-chains, which are similarly linked, also have other chains attached to them (Peat, Whelan & Thomas, 1952). (The single reducing group in the molecule is carried by a C-chain; this can be neglected in the present study.)

Preparation of isoamylase

Acetone fractionation. Dried yeast (200 g.) was extracted at room temperature with 0·1M-sodium bicarbonate solution (1000 ml.) for 2 hr. All subsequent operations were carried out at low temperatures (–4° or –7°). After centrifuging (1500g; 20 min.; –4°), the supernatant solution (580 ml.) was treated with an equal volume of cold A.R. acetone at –7°. The precipitate was collected (centrifuging at –4°), and triturated for several hours with 0·2M-sodium citrate buffer (pH 5·8; 500 ml.). Insoluble material was discarded, and the solution (480 ml.) fractionated with acetone at –7°. The protein fractions precipitated in acetone concentrations 0–20, 20–33 and 33–42% (v/v) were collected, washed with cold acetone, and dried *in vacuo* at 0° over phosphorus pentoxide, giving respectively fractions 1–3. The residue from the first extraction was re-extracted with bicarbonate solution (500 ml.), and to the solution cold acetone to 50% (v/v) concentration was added, giving fraction 4.

The isoamylase activities of the four fractions, expressed as the percentage increase in the β-amylolysis limit of glycogen were 8, 48, 10 and 6 respectively. Fraction 3 contained the greatest amount of yeast branching enzyme and maltase.

Fraction 2 could be used for isoamylolysis without further purification in certain experiments, e.g. the action on glycogen as followed by β-amylolysis or iodine staining. However, in other experiments, the contaminating yeast branching enzyme and maltase interfered, and had, therefore, to be eliminated.

Further purification of isoamylase. (*a*) Elimination of yeast branching enzyme. A starch-adsorption method similar to that developed by Hobson *et al.* (1951) for the removal of α-amylase and Q-enzyme from potato and bean preparations was used. Isoamylase (fraction 2; 5 g.) was dissolved in aqueous 20% ethanol solution (200 ml.) at –4°; anhydrous sodium sulphate (2 g.) was added and the solution stirred slowly with cooling to –7°. Maize starch (112 g.) was added to the solution, and stirring continued for 2 hr. After centrifuging at –4°, cold acetone (180 ml.) was added to the supernatant solution (90 ml.). The precipitate was collected at –4°, washed twice with cold acetone and dried under vacuum. The yield was 2·1 g.

This procedure gave an isoamylase preparation having little or no action on amylose. On incubation with potato amylose, the extinctions (680 mμ) of the iodine complex after 5 min., 2 and 24 hr., were 0·50, 0·50 and 0·41 respectively. The β-amylolysis limit of wrinkled-pea amylose was 75 and 77% respectively before and after treatment with isoamylase (Cowie, Fleming, Greenwood & Manners, 1957).

(*b*) Elimination of maltase. (i) *Acid treatment.* Fraction 2 (450 mg.) was dissolved in 0·2M-sodium acetate buffer (pH 4·5; 45 ml.) at room temperature and samples (5 ml.) were removed at intervals. The relative stabilities of isoamylase and maltase are given in Table 1.

Despite some loss in activity, the isoamylase could be purified by dissolving fraction 2 (1 g.) in buffer, pH 4·5 (100 ml.), for 10 hr. at 18°; after adjusting the pH to 5·9 and centrifuging, the protein (fraction 2*a*) was isolated by precipitation with cold acetone. The yield was 0·55 g.

On incubation of fraction 2*a* with maltose, at 20° and pH 5·9 for 23 hr., no increase in reducing power was observed [with either Somogyi (1952) or Phillips & Caldwell (1951) reagents] and glucose was not detected by paper chromatography. Under similar conditions, the β-amylolysis limit of glycogen and amylopectin β-dextrin increased by 25 and 23% respectively.

(ii) *Further acetone fractionation.* A bicarbonate extract of yeast (100 g.) was treated with acetone as before, except that all the material precipitated by 30% acetone concentration was collected (i.e. approx. fractions 1 and 2). This precipitate was redissolved in 0·2M-sodium citrate buffer (pH 6·0; 100 ml.), insoluble material removed by centrifuging, and the solution again fractionated with acetone. The protein (fraction 5) obtained between 33 and 50% acetone concentration was collected, washed and dried. Yield, 8·0 g.

Fraction 5 had no action on maltose or maltotriose, but slowly hydrolysed isomaltose. On incubation (0·2% solution) with glycogen (0·1% solution) at pH 6·0 and 20°, the extinction of the polysaccharide–iodine complex at 440 mμ increased by 13, 28 and 39% after 2, 4 and 24 hr. respectively.

Table 1. *Stability of isoamylase and maltase at* pH 4·5 *and* 18°

Maltase activity is expressed as apparent hydrolysis of maltose (%). Isoamylase activity is expressed as increase in the β-amylolysis limit of glycogen (%).

Time of incubation (hr.)	Maltase activity	Isoamylase activity
4	40	50
6	31	—
7	22	48
8	6	41
10	0	36

RESULTS

Properties of isoamylase

Effect of enzyme concentration. When digests of glycogen and various amounts of isoamylase were examined by iodine-staining after 2 and 6 hr., a linear relationship between enzyme concentration and the percentage increase in extinction of the polysaccharide–iodine complex was observed.

A linear relationship was also obtained between enzyme concentration and the degree of hydrolysis of glycogen when various amounts of isoamylase were incubated with glycogen and a constant excess of β-amylase. Under these conditions, the extent of β-amylolysis is limited by the concentration of isoamylase.

Effect of temperature and pH. Isoamylase is relatively thermolabile. After incubation at various temperatures for 1 hr., the activity (expressed as the increase in β-amylolysis limit of glycogen) was 15 at 35°, 11 at 40°, 5 at 45°, 4 at 50° and 2 at 55°. These results may be contrasted with those obtained on incubation of fraction 2 with maltose; the relative activity (expressed as the apparent increase in reducing power) was 7·3, 7·1 and 0·1 at 35°, 45° and 55° respectively.

By iodine-staining measurements, the optimum temperature was about 25°, the extinction of a glycogen–iodine complex increased by 0·134, 0·143, 0·142 and 0·122, after incubation at 21, 25, 29 and 37° for 1·5 hr.; after 7 hr., the corresponding figures were 0·142, 0·153, 0·151 and 0·143.

In sodium acetate buffer, activity was optimum at about pH 6·0, as determined by both iodine-staining and β-amylolysis-limit methods.

In B.D.H. Universal buffer (British Drug Houses Ltd.) (see later) isoamylase was inactive.

Effect of various inhibitors and activators. In the following experiments, the action on glycogen was followed by iodine-staining, and the results are expressed as the percentage increase in extinction at 460 mμ.

(*a*) Ammonium molybdate. This substance, which inhibits R-enzyme (Whelan, 1958), also caused a marked reduction in isoamylase activity. In the presence of 0, 0·2 and 2·0% (w/v) of ammonium molybdate, the activities after 4 hr. were 78, 19 and 4 respectively, and, after 24 hr., 95, 41 and 8.

(*b*) Other inorganic salts. The effect of various salts which alter the activity of other starch-metabolizing enzymes (e.g. α- and β-amylase, Q-enzyme) was examined. The results are shown in Table 2, and suggest that sodium borate is the inhibiting component of B.D.H. Universal buffer.

(*c*) Gluconolactone. This lactone has a pronounced effect on many carbohydrases (Conchie & Levvy, 1957; Reese & Mandels, 1959) and, as shown in Table 2, completely inhibits isoamylase at a concentration of 10 mM.

(*d*) Sulphydryl reactants. Isoamylase action was only partly inhibited by SH-group reactants as shown in Table 3.

Specificity of isoamylase

The partially purified isoamylase preparation (fraction 5) had no hydrolytic activity on the α-(1 → 4)-glucosidic linkages in maltose (by reducing-power measurements and paper chromatography), in maltotriose (by paper chromatography), or in amylose (by iodine-staining and β-amylolysis). On incubation with glycogen the increase in reducing power was extremely small; in one experiment this corresponded to about 7% apparent conversion into maltose.

Action on α-(1 → 3)-*glucosidic linkages.* Isolichenin, a linear polysaccharide containing 40% of α-(1 → 4)- and 60% of α-(1 → 3)-glucosidic linkages (Chanda, Hirst & Manners, 1957), was treated with salivary α-amylase as follows: isolichenin (47·5 mg.), α-amylase (5 mg.; 200 units), 0·1 M-sodium chloride (5 ml.) and water (to 25 ml.) were incubated at 35° for 24 hr. The increase in reducing power corresponded to 9·7% apparent conversion into

Table 2. *Effect of salts and gluconolactone on isoamylase activity*

Digests containing glycogen (25 mg.), isoamylase (50 mg.), 0·2 M-sodium acetate buffer (pH 6·0; 5 ml.) and inhibitor (1 ml.) in a total volume of 10 ml. were incubated at 20° for 15 hr.

Inhibitor	Final concn. (mM)	Activity (increase in extinction, %)
—	—	54
Mercuric chloride	0·1	1
Magnesium chloride	20	20
Sodium borate	10	1
Sodium chloride	20	17
Sodium fluoride	10	33
Gluconolactone	5	28
Gluconolactone	10	1

Table 3. *Effect of sulphydryl reactants on isoamylase activity*

The composition of the digests was as in Table 2, except that the final volume was 25 ml. Samples (2·5 ml.) were removed initially and after (*a*) 2·5 hr. and (*b*) 22 hr. for analysis.

Conditions	Concn. (mM)	Activity (increase in extinction, %) (*a*)	(*b*)
Control	—	22	34
Iodoacetate	10	17	25
Phenylmercuric acetate	0·01	10	20
p-Chloromercuribenzoate	0·01	9	24

glucose. Part of the solution (13 ml.) was then incubated with isoamylase (60 mg.) and buffer (4 ml.). After 24 hr. at 20° the reducing power had not increased. It is concluded that isoamylase has no action on α-(1 → 3)-linkages.

Action on β-(1 → 6)-glucosidic linkages. Pustulan, an insoluble linear polysaccharide composed of β-(1 → 6)-glucosidic linkages (Lindberg & McPherson, 1954) was partially hydrolysed (2 %, w/v) in N-sulphuric acid at 98° for 135 min.). After cooling, neutralization (barium carbonate), and filtration, the filtrate was concentrated *in vacuo* at 35°, and shown by paper chromatography to contain a regular series of oligosaccharides, together with glucose. Part of the concentrate (≡ 40 mg. of pustulan) was incubated with isoamylase (60 mg.) and buffer (3 ml.) in a final volume of 25 ml. No change in reducing power was observed during incubation at 20° for 24 hr. Hence, isoamylase has no action on β-(1 → 6)-glucosaccharides.

Action on glycogen. Incubation of isoamylase with glycogen samples from a variety of biological sources caused a marked increase in β-amylolysis limit ranging from 10 to 32 %. The results are shown in Table 4. Isoamylolysis is considered to be complete for two reasons: first, the increase in the β-amylolysis limit was the same after incubation with isoamylase for 24, 48 or 72 hr.; secondly, addition of glycogen to a 72 hr. digest of isoamylase and glycogen caused an increase in β-amylolysis limit from 44 to 51 %, thus showing that active isoamylase was still present.

The product of the action of isoamylase on a sample of oyster glycogen was then examined in detail; for convenience this will be referred to as 'isodextrin' but this does not necessarily indicate a limit-dextrin configuration.

Table 4. *Effect of isoamylase on the β-amylolysis limit of polysaccharides*

β-Amylolysis limits were measured before (*b*) and after (*a*) treatment with isoamylase at pH 5·9 and 18° for 18–24 hr.

	Average chain length*	β-Amylolysis limit (%) (*b*)	(*a*)
Glycogen sample			
Helix pomatia	7	37	60
Human liver	14	46	60
Human kidney	14	46	59
Oyster	10	40	65
Rabbit liver (IV)	13	46	78
(V)	12	52	76
(X)	12	49	59
Synthetic†	14	47	75
Trichomonas foetus	15	60	80
Yeast (brewer's)	13	44	68
Commercial	13	45	65
Amylopectin sample			
Dunaliella bioculata	15–16	61	76
Potato I	22	53	80
II	21	61	77
Protozoal	22	63	80
Synthetic‡	—	57	82
Waxy-maize starch	20	50	66
Waxy-maize starch β-dextrin	10	0	71
Waxy-sorghum starch	22	52	76

* Determined by periodate oxidation.

† Prepared by the action of yeast branching enzyme on amylopectin (Gunja, Manners & Khin Maung, 1960).

‡ Prepared by the action of yeast branching enzyme on amylose (Gunja *et al.* 1960).

Preparation and properties of glycogen isodextrin

Glycogen (1 g.) was incubated with isoamylase (fraction 2; 100 mg.), buffer (pH 5·9; 10 ml.) and water (30 ml.) at 21° for 48 hr. The enzyme was then inactivated by heating (15 min. at 98°). After removal of coagulated protein by filtration (G. 3 filter), the solution was dialysed against distilled water at room temperature for 48 hr., and the isodextrin isolated by freeze-drying. Yield, 0·85 g.

Isodextrin (147 mg.) and glycogen (255 mg.) were oxidized with a suspension of potassium metaperiodate (110 ml.) at room temperature, as described by Gunja *et al.* (1960). The production of formic acid was constant after 10 days and amounted to 2·33 and 2·80 mg. respectively. These figures correspond to average chain lengths of 12·0 and 10·0 glucose residues. The isodextrin had β-amylolysis limits of 50 and 58 % before and after treatment with isoamylase, and the iodine complex showed maximum absorption at 435–440 mμ. For the original glycogen, the corresponding figures were 40 % and 420 mμ.

Stepwise action of isoamylase and β-amylase on glycogen

Yeast glycogen (25 mg.) was incubated with isoamylase (40 mg.) in a total volume of 13 ml. at pH 5·9 and 20° for 24 hr. After heat inactivation, β-amylase was added, and, after 24 hr., the β-amylolysis limit was 67 %. The β-amylase was then inactivated by heating, and isoamylase solution (40 mg. in 1 ml. of water) added. This treatment caused only a small increase in the reducing power of the digest (from 17·6 to 18·4 mg. of apparent maltose) but the β-amylolysis limit of the polysaccharide increased to 81 %. Removal of the outer chains of the glycogen isodextrin had thus facilitated further isoamylolysis.

Action of isoamylase on amylopectin and amylopectin β-dextrin

The β-amylolysis limits of various amylopectin samples determined before and after incubation with isoamylase are shown in Table 4.

Amylopectin isodextrin was prepared from potato amylopectin (1 g.) by a method similar to that described for glycogen isodextrin, and, as before, the product was not a limit-dextrin. Yield, 0·6 g. Amylopectin isodextrin had β-amylolysis limits of 45 and 80 % before and after treatment with isoamylase, and the iodine complex showed maximum absorption at 550 mμ. The limiting viscosity numbers for potato amylopectin and the isodextrin were 205 and 41 respectively.

Incubation of amylopectin β-dextrin with isoamylase (fraction 2) failed to produce the expected increase in iodine-staining power (see Discussion). In one experiment, the extinctions (at 540 mμ) of the polysaccharide–iodine complexes after isoamylolysis for 0·2, 1, 2 and 24 hr. were respectively 0·11, 0·12, 0·12 and 0·11. The same enzyme preparation increased the extinction of the iodine complex of a glycogen from 0·055 to 0·079 within 1 hr. However, the β-amylolysis limit of amylopectin β-dextrin always increased.

With amylopectin β-dextrin and an acid-treated isoamylase preparation, maltose and maltotriose were produced (paper chromatography). This provides a further indication that the A-chain stubs in an amylopectin β-limit dextrin contain two or three glucose residues (cf. Peat *et al.* 1952).

Simultaneous action of isoamylase and β-amylase on polysaccharides

Since isoamylase causes a greater increase in the β-amylolysis limit of amylopectin β-dextrin than of amylopectin (Table 4), it was possible that isoamylolysis was being hindered by the presence of long outer chains. The simultaneous action of iso- and β-amylase on various polysaccharides was therefore examined, as, under these conditions, exterior chains would be reduced to stubs of only two to three glucose residues. Polysaccharides (15–25 mg.), isoamylase (40 mg.), β-amylase (1000 units), buffer (4 ml.) and water to 25 ml. were incubated at 20° for 24 hr. The following conversions (%) into maltose were obtained: potato amylopectin, 84; waxy-maize starch, 83; oyster glycogen, 95; synthetic glycogen (see Gunja *et al.* 1960), 90. In a control digest containing maltose, no increase in reducing power was observed. It follows that very extensive degradation of the polysaccharides had occurred.

Action on isomaltose. Paper-chromatographic analysis showed that fraction 2 slowly hydrolysed isomaltose to glucose (Manners & Khin Maung, 1955) and it was concluded that isoamylase could attack terminal α-(1 $\rightarrow$ 6)-glucosidic linkages. However, more recent evidence suggests that isoamylase has no action on isomaltose.

Isomaltose (7·7 mg.), buffer (5 ml.), gluconolactone (4·5 % solution; 0, 0·5 or 1·0 ml.) and isoamylase (25 mg.) were incubated in a total volume of 25 ml. at 20°. After 24 hr., the apparent increases in maltose equivalents were 1·2, 1·0 and 0·0 respectively, and at 72 hr., 1·9, 1·8 and 0·0. The isomaltase activity is therefore completely inhibited by 10 mM-gluconolactone but is little altered in the presence of 5 mM-inhibitor, whereas isoamylase activity is reduced by 56 % under these conditions.

The non-identity of yeast isomaltase and isoamylase has been confirmed by the separation of these two activities by chromatography on alumina or continuous paper electrophoresis (Gunja, 1959) and independently by Kobayashi (1958) using starch-gel electrophoresis.

Action on α-dextrins. Maltodextrin (10 mg.), isoamylase (21 mg.), buffer (pH 5·9; 1 ml.) and water to 5 ml. were incubated at 20° for 24 hr. After heating and cooling, the β-amylolysis limit was determined. The result, 94 %, may be compared with that of the original maltodextrin, 67 %. Most of the α-(1 $\rightarrow$ 6)-linkages in the maltodextrin were therefore hydrolysed.

DISCUSSION

The present study shows that extracts of brewer's yeast contain a carbohydrase, isoamylase, which hydrolyses a proportion of the α-(1 $\rightarrow$ 6)-glucosidic inter-chain linkages in both glycogen and amylopectin. In this respect it is similar to the debranching enzyme in black-koji mould (Ueda, 1957), but clearly differs from the corresponding plant and animal debranching enzymes, R-enzyme and amylo-(1 $\rightarrow$ 6)-glucosidase.

Although extensive purification of isoamylase has not been attempted, acetone fractionation of yeast extracts has consistently given preparations with activity sufficient to enable the action pattern and some of the properties of the enzyme to be determined. For example, isoamylase action is optimum at about pH 6 and 25° [cf. the properties of amylosynthease reported by Nishimura & Minagawa (1931)] and, unlike β-amylase, does not need SH groups for activity.

The available evidence shows that isoamylolysis of amylopectin and glycogen is incomplete since some 10–20 % is resistant to the successive actions of isoamylase and β-amylase (see Table 4). The inability of isoamylase to hydrolyse all the inter-chain linkages appears to be due to steric hindrance caused by the exterior portions of the B-chains in the interior of the molecules. This is shown by the greater degradation caused by the simultaneous action of these two enzymes. This situation is similar to that observed during R-enzyme action on amylopectin and amylopectin β-dextrin (Hob-

son *et al.* 1951); these polysaccharides then had β-amylolysis limits of 74 and 73 % respectively, the latter corresponding to 87 % degradation of the original amylopectin.

Evidence that isoamylase removes a large proportion of A-chains is shown by the end-group assay of glycogen isodextrin. Assuming the presence of equal numbers of A- and B-chains in a glycogen of chain length 10 and β-amylolysis limit 40 %, the average lengths of the A- and B-chains are 6·5 and 13·5 glucose residues respectively. Hydrolysis of A-chains, and subsequent removal by dialysis, will therefore cause an overall increase in average chain length; experimentally, the increase was two glucose residues. This evidence is also confirmed by comparison of the observed β-amylolysis limit after isoamylase treatment with that calculated on the assumption that all the A-chains have been removed. For yeast glycogen the figures are 68 and 68 % respectively, and for potato amylopectin, 80 and 75 %.

The action of isoamylase on amylopectin produces a marked decrease (80 %) in viscosity, and hence in molecular weight, and an increase in β-amylolysis limit. The general conclusions of Maruo & Kobayashi (1951) are therefore substantiated, and a similarity with the action of R-enzyme on amylopectin is established (Hobson *et al.* 1951). However, isoamylolysis did not increase the iodine-staining power, as expected; it is probable that the presence of branching-enzyme impurity would effectively mask such changes (cf. Gunja *et al.* 1960), although, since yeast branching enzyme has no action on glycogen, measurement of activity towards the latter substrate is not affected. In the experiments of Kobayashi (1956), the activity of isoamylase could be followed by measurement of the increase in iodine coloration (at 620 mμ) of glutinous rice starch.

Since isoamylase appears to have no action on α-(1 $\rightarrow$ 3)-, α-(1 $\rightarrow$ 4)- or β-(1 $\rightarrow$ 6)-glucosidic linkages, it provides a useful tool for the structural analysis of starch-type polysaccharides. Examples of its use (see Table 4) include the characterization of inter-chain linkages in protozoal and algal starches, which are not available in the quantity required for examination by methylation or periodate oxidation techniques. Nevertheless, the conclusion on specificity must be considered with caution. The failure of a carbohydrase to attack a particular linkage in an oligosaccharide does not necessarily imply that the same linkage in a polysaccharide would also be resistant. Thus R-enzyme will not hydrolyse isomaltose, but acts on amylopectin. Substrates containing for example both β-(1 $\rightarrow$ 6)- and α-(1 $\rightarrow$ 4)-glucosidic linkages are not available for examination. We have therefore concluded that it is unlikely that isoamylase has any action on linkages other than the non-terminal α-(1 $\rightarrow$ 6)-glucosidic type as present in starch-type polysaccharides.

The incomplete degradation of waxy-maize starch and potato amylopectin by the combined action of iso- and β-amylase has structural significance. This may indicate the presence of linkages other than the α-(1 $\rightarrow$ 4)- or α-(1 $\rightarrow$ 6)-glucosidic type. Residues of glucose 6-phosphate are possible resistant structures (cf. Posternak, 1951). Alternatively, the presence of a small proportion of adjacent α-(1 $\rightarrow$ 6)-linked glucose residues [which give rise to doubly and triply linked α-dextrins on α-amylolysis (see Roberts & Whelan, 1960)] may prevent iso- and β-amylolysis.

The presence of a debranching enzyme in yeast extracts has been shown independently by Hopkins (1955, 1958) and Hopkins & Kulka (1957*a*, *b*) during a study of dextrin fermentation. With amylopectin β-dextrin as substrate, and in the presence of β-amylase, various yeast preparations caused a decrease in iodine-staining power. Enzyme action was, however, incomplete, about two-thirds of the β-dextrin being resistant. In the absence of β-amylase, the iodine-staining power increased. Possible activity towards glycogen was not reported. Some differences in the debranching enzymes from *Saccharomyces diastaticus* and *S. cerevisiae* were noted; thus the former activity was the more stable at pH 4·6.

The ability of fraction 2 to cause an increase in the β-amylolysis limit of maltodextrin would suggest that isoamylase also possesses 'limit-dextrinase' activity. MacWilliam & Harris (1959) have shown that preparations of R-enzyme that attack the α-(1 $\rightarrow$ 6)-linkages in both amylopectin and α-limit dextrin can be separated into two specific enzymes, one acting only on amylopectin and the other only on α-limit dextrin. The possibility that the yeast limit-dextrinase activity is caused by a separate carbohydrase is therefore now being examined.

In all the above experimental work, a brewer's yeast was used. However, extraction of a baker's yeast with bicarbonate followed by fractionation with acetone has also given preparations showing isoamylase activity. For example, the protein precipitated by 0–20, 20–33 and 33–42 % concentration of acetone increased the β-amylolysis limit of glycogen by 5, 24 and 23 % respectively (Gunja, 1959).

SUMMARY

1. Extracts of brewer's yeast contain a debranching enzyme, isoamylase, which may be isolated by acetone fractionation at $-7°$.

2. Enzyme action on glycogen is optimum at about pH 6 and 25°, is inhibited by borate (10 mM)

and mercuric chloride (0·1 mM) but not by sulphydryl reactants.

3. The reaction is characterized by a marked increase in iodine-staining power and β-amylolysis limit but α-(1 → 4)-glucosidic linkages in glycogen are not attacked. Enzyme action is incomplete, only the outer α-(1 → 6)-glucosidic linkages being hydrolysed.

4. Isoamylase differs from the plant and animal debranching enzymes [R-enzyme and amylo-(1 → 6)-glucosidase] since both amylopectin and glycogen are substrates.

5. Purified isoamylase has no action on maltose, isomaltose or maltotriose.

6. Isoamylase is also present in baker's yeast.

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The Metabolism of [4-^{14}C]Progesterone in the Cat: Biliary and Urinary Excretion of Conjugated Metabolites

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Marrian (1949, 1954) stressed the need for a systematic study of progesterone metabolism in order to account for the large proportion of that hormone which is not excreted in the urine, by man and some animals, as pregnanediol (5β-pregnane-3α:20α-diol) and closely related substances. The availability of steroid hormones labelled with ^{14}C and ^{3}H now permits such systematic studies to be carried out, and some investigations with labelled progesterone have been reported. When [21-^{14}C]-