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Studies on Carbohydrate-Metabolizing Enzymes

6. THE ACTION OF POTATO PHOSPHORYLASE (P-ENZYME) ON STARCH-TYPE POLYSACCHARIDES*

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Although the synthetic action of P-enzyme (potato phosphorylase) has been widely studied (for review see Whelan, 1958), observations on the degradation of amylose, amylopectin and glycogen by this enzyme are limited, and the results are often conflicting. For example, Katz, Hassid & Doudoroff (1948) claimed that potato amylose was almost completely converted into α -D-glucosyl phosphate by P-enzyme, whereas Peat, Thomas & Whelan (1952) found only 70% conversion. Baum, Gilbert & Scott (1956) have suggested that the incomplete phosphorolysis was due to oxidative modification of the substrate during the preparation from starch; other explanations, are, however, possible.

The action of P-enzyme on amylopectin, like that of β -amylase, is confined to the exterior chains, but the extent of degradation (see Table 1) is uncertain, and there appears to be no constant difference between the phosphorolysis and β -

amyolysis limits. The extent of arsenolysis of amylopectin (see equation 2) was found by Meyer, Weil & Fischer (1952) to be the same as the β -amyolysis limit.

This would suggest that P-enzyme can, in fact, degrade α -1 $\rightarrow$ 4-glucosidic linkages which are separated by only two or three glucose residues from an α -1 $\rightarrow$ 6-glucosidic inter-chain linkage. However, the β -amyolysis limits reported by Meyer *et al.* (1952) are about 10% higher than those found by other workers.

The extent of P-enzyme action on glycogen is not known. Swanson (1948) found that a sample of rabbit-liver glycogen had a phosphorolysis limit of

* Part 5: Gunja & Manners (1960).

Table 1. *Extent of degradation of amylopectins by P-enzyme and β -amylase*

Source of amylopectin	Phosphorolysis (%)	Arsenolysis (%)	β -Amylolytic (%)	Reference
Corn	40*	—	60	Swanson (1948)
Potato	59†	54	About 55	Katz, Hassid & Doudoroff (1948)
Potato	39†	—	53	Bourne, Sitch & Peat (1949)
Potato‡	48†	—	59	Peat, Thomas & Whelan (1952)
Potato	—	70	70	Meyer, Weil & Fischer (1952)
Tapioca	57†	—	—	Katz <i>et al.</i> (1948)
Tapioca	—	62	62	Meyer <i>et al.</i> (1952)
Waxy-maize starch	—	70	70	Meyer <i>et al.</i> (1952)

* Calculated from residual non-dialysable polysaccharide.

† Conversion into α -D-glucosyl phosphate.

‡ This sample was a sub-fraction and was considered to possess unusual properties.

Table 2. *Action of P-enzyme on amylopectins*

Sample	Average chain length*	P-limit (%)	β -Amylolysis limit (%)	Exterior chain length†
Waxy-maize starch	22	45	58	15
Waxy-sorghum starch	20	33	56	13-14
Potato amylopectin	24	43	57	16

* Determined by periodate oxidation.

† No. of glucose residues removed by β -amylase plus 2.5.

20 %, whereas Fischer & Hilpert (1953) reported that crystalline P-enzyme had almost no action on oyster or muscle glycogen.

In contrast with the above work, in which amylopectins or glycogens of unreported chain length were used, we have examined the phosphorolysis of various glycogen and amylopectin samples of known chain length. In certain instances the β -amylolysis limit and the extent of degradation by muscle phosphorylase (ϕ -limit) were also known, so that detailed comparisons of action pattern could be made. The effect of oxygen treatment on the phosphorolysis of amylose has also been examined. Preliminary accounts of this work have been published (Liddle & Manners, 1955; Liddle, 1956; Wright, 1960).

METHODS AND MATERIALS

Analytical methods. Inorganic phosphate and α -D-glucosyl phosphate were determined by the methods described by Liddle & Manners (1957*a*). The conditions for β -amylolysis of glycogen and amylopectin were those of Liddle & Manners (1957*b*), and for amylose (at pH 3.6) those of Cowie, Fleming, Greenwood & Manners (1957); iodine-staining and viscosity measurements were carried out as by Gunja, Manners & Khin Maung (1960).

Substrates. The glycogen samples were those described by Liddle & Manners (1957*a, b*). Waxy-maize and sorghum starches were commercial products; Dr C. T. Greenwood kindly provided a potato (var. Epicure) amylopectin of known chain length and β -amylolysis limit, and Dr J. R. Turvey a sample of *Zea mays* phytoglycogen A (see Peat, Whelan & Turvey, 1956). Linear amylose was extracted from potato (var. Kerr's pink) starch and maize starch (sample I) by controlled aqueous leaching at 60° and precipitated with butan-1-ol. Aqueous leaching of maize starch at higher temperatures gave subfractions (II, III and IV) containing anomalous structures (see Table 4). The degree of polymerization (DP) was calculated from the limiting viscosity number $[\eta]$ in m -KOH from the relationship $DP = 7.4[\eta]$ (Cowie & Greenwood, 1957).

Enzyme preparations. P-Enzyme was prepared from potatoes, either by the method of Baum & Gilbert (1955) to the stage before crystallization involving fractionation of potato juice with ethanol in the presence of amylose, or by a method developed by Dr D. French and Dr H. B. Wright using ammonium sulphate to fractionate heat-treated potato juice. We are grateful to Dr G. A. Gilbert and to Dr Wright for supplying unpublished data. Possible contaminants in P-enzyme preparations include α -amylase, Q-enzyme, R-enzyme, D-enzyme and phosphatase(s). The

last two activities could not be detected; α -amylase and Q-enzyme could be selectively inhibited, when present, by mercuric chloride (30 mM), and R-enzyme by ammonium molybdate (0.2 %, w/v) and one or both inhibitors were added to the polysaccharide-P-enzyme digests (cf. Bailey, Thomas & Whelan, 1951). The unit of P-enzyme activity is that defined by Green & Stumpf (1942).

Phosphorolysis experiments. Digests were prepared containing polysaccharide (about 20 mg.), 0.5 M-sodium phosphate buffer (pH 6.0; 2 ml.), P-enzyme (about 7 units), inhibitors (1 or 2 ml.) and water to a final vol. of 10 ml. After incubation at 35° in the presence of toluene, samples (2 ml.) were removed at intervals for the estimation of α -D-glucosyl phosphate. With 0.3 unit of P-enzyme/mg. of amylopectin [conditions similar to those used by Peat *et al.* (1952)], phosphorolysis was complete within 24 hr. Assuming 50 % phosphorolysis, the ratio inorganic phosphate:ester phosphate is about 15:1, a value greatly in excess of that (6.7:1) at the equilibrium of the phosphorolytic reaction at pH 6.0 (Hanes, 1940).

RESULTS

Action on amylopectin

Waxy-maize starch was rapidly degraded on incubation with P-enzyme. Under standard conditions, conversions into α -D-glucosyl phosphate after 2, 4 and 24 hr. were 31, 38 and 45 % respectively. On addition of fresh P-enzyme to the digest (final concentration 0.55 unit/mg.) no further phosphorolysis occurred within 24 hr., indicating that enzyme action was complete. The results obtained with two other amylopectin samples are recorded in Table 2.

Action on glycogen

Under the standard conditions, the rate of phosphorolysis of glycogen (from *Mytilus edulis* VI) was extremely slow, and enzyme action was incomplete. After 2, 4 and 24 hr. the degradation was 6, 7 and 9 % respectively, and on increasing the enzyme concentration (to 0.57 unit/mg.) the degree of phosphorolysis increased to 11 % after a further 24 hr. Although the conditions of phosphorolysis were then varied, a true limit could not be obtained. For example, in two digests containing (a) glycogen (1.3 mg./ml.) and P-enzyme (0.83 unit/ml.), (b) glycogen (1.0 mg./ml.) and P-enzyme (0.62 unit/ml.), the conversion into α -D-glucosyl phosphate was (a) 14, (b) 16 % after 96 hr.

The rate of phosphorolysis was not appreciably altered by increasing the relative enzyme concentration from 0.3 to 0.6 unit/mg. of glycogen. Similar results were obtained with glycogen from various biological sources, and P-enzyme prepared by either of the two methods, and are summarized in Table 3. However, Fischer & Hilpert (1953) and W. J. Whelan (personal communication) were unable to demonstrate any phosphorolysis of other glycogen samples.

Action on amylose and oxygen-treated amylose

Peat *et al.* (1952) reported that the action of both β -amylase and P-enzyme on potato amylose ceased at about 70 % conversion into maltose and α -D-glucosyl phosphate respectively; this indicates the presence of a small number of anomalous structures in the amylose. Cowie *et al.* (1957) showed that potato amylose was heterogeneous with respect to both molecular structure and size. By aqueous leaching an amylose fraction (40 % by weight) with DP 1800 and β -amylolysis limit 99 % could be obtained. The residual amylose had DP about 6000 and β -amylolysis limit 50 % and apparently contained all the anomalous structures.

The controlled aqueous leaching of starch thus yields an ideal substrate for examining the effect of oxygen treatment.

From maize starch the amylose had DP 1070 and β -amylolysis limit 98 %, and from potato starch DP 2150 and β -amylolysis limit 99 %. Both samples were therefore devoid of anomalous structures.

The maize amylose was heated in 0.5N-sodium hydroxide solution in an atmosphere of oxygen for 20 min. at 98°. The product isolated as the butan-1-ol complex had DP 145 and a β -amylolysis limit of 87 %. This treatment had therefore caused considerable degradation of the amylose molecule and also introduced some 'barriers' to β -amylase. On incubation with P-enzyme (with 0.6 unit/mg. of amylose) the iodine-staining power of the substrate ($E_{680\text{ m}\mu}$) fell by 81 %, and the conversion into α -D-glucosyl phosphate was 73 %. By contrast, P-enzyme caused a 97 % decrease in the extinction of the untreated amylose. Barriers to P-enzyme action had therefore also been introduced.

The experiment was repeated with potato amylose, but with milder conditions of oxygen treatment. Enzyme action was followed by measure-

Table 3. *Action of P-enzyme on glycogens*

Glycogen sample	Average chain length*	Phosphorolysis (%)†	β -Amylolysis limit (%)	Exterior chain length	ϕ -Limit (%)
Rabbit liver I	13	3	25	5-6	14
Rabbit liver V	14	11	51	9-10	32
<i>Helix pomatia</i> II	7	5	37	4	20
Human liver	14	16	46	9	—
<i>Mytilus edulis</i> VI	13	9	46	8-9	28
Phytoglycogen A	13	19	50	9	—
<i>Trichomonas foetus</i>	15	17	60	11-12	31
Yeast (brewer's)	13	21	44	8	31

* Determined by periodate oxidation.

† Conversion into α -D-glucosyl phosphate after incubation for 24-48 hr., with 0.3-0.9 unit/mg. of glycogen.

Table 4. *Measurement of degradation of amylose by iodine-staining and other methods*

Sample	Period of degradation (hr.)	Fall in $E_{680\text{ m}\mu}$ (%)	Conversion into α -D-glucosyl phosphate (%)
Phosphorolysis			
Maize amylose II	4	85	75
	22	95	85
	48	95	85
Maize amylose III	24	95	82
β -Amylolysis			
			Conversion into maltose (%)
Maize amylose IV	6	93	82
Maize amylose IV (after oxygen treatment)*	6	88	77

* 20 min. in 0.5M-potassium hydroxide at 98°.

Table 5. *Properties of amylose samples*

Sample	Conditions of treatment			[η]	P-limit (%) [*]
	Atmosphere	pH	Time (min.)		
Initial	—	—	—	290	98
1	Oxygen	9.0	30	240	83
2	Nitrogen	9.0	30	280	97
3	Oxygen	7.0	30	260	88†
4	Oxygen	Water	180	250	91
5	Oxygen	9.0	70	—	92

^{*} Digests contained 10–12 mg. of amylose and 5–6 units of P-enzyme in a total volume of 10 ml.

† β -Amylolytic limit, 94%.

ment of the α -D-glucosyl phosphate liberated rather than by the facile iodine-staining method. The latter will give erroneous results, as shown in Table 4, since the iodine complexes of short-chain amyloses (DP < 50) produced by multi-chain degradation (cf. Whelan & Bailey, 1954) towards the later stages of the reaction do not show maximum absorption at 680 m μ . [The iodine-staining method as applied to β -amylolysis also gives higher results than that from measurement of the production of maltose (Cowie *et al.* 1957).]

An aqueous solution of potato amylose was divided into five equal portions (about 100 mg. of polysaccharide in 25 ml. of water), B.D.H. (British Drug Houses Ltd.) Universal buffer (a mixture of sodium borate, phenylacetate and phosphate), pH 7.0 or 9.0, or water (10 ml.) added and heated to 98° for varying times in the presence of oxygen or nitrogen. The amylose samples were then twice precipitated as the butan-1-ol complex. The limiting viscosity numbers and phosphorolysis limits are recorded in Table 5.

The results show that even a 30 min. treatment of amylose in oxygen at pH 7.0 has a marked effect on the phosphorolysis limit and limiting viscosity number; at pH 9.0 the effect is greater. In the presence of nitrogen there is no significant change in either molecular size or phosphorolysis limit.

DISCUSSION

Phosphorolysis of amylopectin and glycogen

The present studies show that amylopectin is rapidly attacked by P-enzyme with the production of 33–45% of α -D-glucosyl phosphate and a residual dextrin (P-dextrin). This is in good agreement with the results of Bourne, Sitch & Peat (1949). The smallest linear maltosaccharide that is readily degraded by P-enzyme is maltopentaose (W. J. Whelan & J. M. Bailey, personal communication); however, calculation shows that the exterior chain 'stubs' in the P-dextrin contain about six glucose residues. The presence of α -1 $\rightarrow$ 6-glucosidic inter-chain linkages clearly represents a considerable

hindrance to the P-enzyme-substrate reaction. This contrasts with β -amylolysis, where the exterior chain 'stubs' in a β -dextrin probably contain two or three glucose residues, and maltotriose is slowly attacked.

The degradation of glycogen by P-enzyme is slow and is limited to 3–21% conversion into α -D-glucosyl phosphate, corresponding to the loss, on the average, of only 0.5–3.0 glucose residues/chain. It follows that only a proportion (presumably the longest) of the exterior chains are attacked. The resistance of glycogen to extensive P-enzyme action may be correlated with the relatively short outer chain (about 8 glucose residues for many glycogens, compared with about 16 for amylopectin) and with the compact nature of the interior of the molecule, which must prevent penetration of the enzyme. The latter factor has been used to explain the inability of R-enzyme to attack most glycogen samples (Peat, Whelan, Hobson & Thomas, 1954).

The results in Table 3 emphasize a little-known difference between plant and animal phosphorylases, since the latter catalyses a rapid degradation of glycogen to give 20–40% conversion into α -D-glucosyl phosphate. Further, a true limit dextrin (ϕ -dextrin) can be obtained, and enzyme action apparently ceases when 'stubs' of four glucose residues have been produced (Walker & Whelan, 1960). P-enzyme and muscle phosphorylase also differ in regard to primer specificity, requirement for activators, molecular weight and chemical composition (Lee, 1960; Manners, 1961), although both enzymes catalyse the same basic reaction.

It is concluded that P-enzyme, unlike β -amylase and muscle phosphorylase, cannot be used as a structural tool for the analyses for glycogen-type polysaccharides. Nevertheless, the degree of degradation of a polysaccharide by P-enzyme may enable a glycogen-type polysaccharide to be differentiated from one of amylopectin-type. For example, Floridean starch is readily degraded (28–35%) by P-enzyme (Fleming, Hirst & Manners, 1956), whereas the rate and extent of degradation

Fig. 1. Degradation of modified amylose by P-enzyme and β -amylase. $\circ$, α -1 $\rightarrow$ 4-Linked glucose residue; $\bullet$, α -1 $\rightarrow$ 4-linked oxidized glucose residue; $\uparrow$, linkage susceptible to β -amylase; R, linkage resistant to β -amylase; $\downarrow$, linkage susceptible to P-enzyme; r, linkage resistant to P-enzyme.

4. The action patterns of P-enzyme and β -amylase are compared.

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The Occurrence of Methyl Ethers of Rhamnose and Fucose in Specific Glycolipids of Certain Mycobacteria

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Individual strains of mycobacteria may produce a large number of complex lipids and it is therefore of interest, from the point of view of strain identification, to inquire whether or not certain of these lipids are produced only by organisms of a particular type or species.

Randall & Smith (1953) isolated a previously

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unknown lipid from three strains of *Mycobacterium bovis* by adsorption chromatography. The substance was characterized by a distinctive infrared spectrum and was shown in this way to be absent from nine strains of *Mycobacterium tuberculosis* (see also Smith, Harrell & Randall, 1954). Moreover, Randall & Smith (1953) found that the lipids of two strains of *M. tuberculosis* could be distinguished one from the other by the combined techniques of