591. *The Enzymic Synthesis and Degradation of Starch. Part VIII. The Use of Mixtures of P- and Q-Enzymes in the Synthesis of Starch-type Polysaccharides.*

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Different mixtures of stock samples of the P- and Q-enzymes of the potato have been used to catalyse the synthesis of starch-type polysaccharides from glucose-1 phosphate. The properties of the polysaccharide products have been shown to be related to the relative proportions of the two enzymes. When a large excess of P-enzyme was employed amylose was produced. A large excess of Q-enzyme gave rise to amylopectin. The polysaccharides synthesised by other mixtures of P- and Q-enzymes appeared to have molecular structures intermediate between those of amylose and amylopectin.

It was suggested in Part I of this series (Bourne and Peat, *J.*, 1945, 877) that the relative proportion of the linear and branched components in a starch polysaccharide synthesised from glucose-1 phosphate by the joint action of phosphorylase (P-enzyme) and the branching factor (Q-enzyme) may be controlled by the ratio of the amounts of the two enzymes employed. Thus an enzyme mixture rich in P-enzyme was expected to give rise to a polysaccharide which was predominantly amylose, whereas a larger proportion of Q-enzyme was believed to favour the formation of amylopectin. By using mixtures of P- and Q-enzymes containing increasing proportions of the latter, it has in fact been possible to synthesise a number of polysaccharides with properties ranging from those of natural amylose to those of amylopectin. The scale of the work was sufficiently large to enable the polysaccharide products to be isolated, purified, and characterised by the methods detailed in our earlier communications.

It was important that only one sample of each enzyme should be employed for the syntheses, a limitation necessitating the isolation of P- and Q-enzymes in stable form so that the requisite stocks could be accumulated. Appropriate methods for the preparation and purification of the enzymes were developed and are described in Part VI (*J.*, 1950, 84).

After a series of preliminary tests designed to reveal the P/Q ratios best suited for the synthesis, from glucose-1 phosphate, of a well-distributed range of polysaccharides, five digests, having the compositions shown in Table III, were incubated at pH 7·1 and 25°, these conditions being imposed by the requirements of the more sensitive enzyme, namely Q-enzyme (Part IV, *J.*, 1949, 1705). In view of the fact that the chain-length of a polysaccharide synthesised by P-enzyme alone is influenced by the ratio of primer chains to glucose-1 phosphate molecules (Sumner, Somers, and Sisler, *J. Biol. Chem.*, 1944, **152**, 479), the only ingredient of the five digests which was varied in amount was *active* Q-enzyme. The overall quantity of Q-enzyme solution was kept the same in each digest, variation in the amount of active enzyme being attained by mixing appropriate volumes of active and heat-inactivated Q-enzyme solutions. No carbohydrate primer was added; sufficient was already present (probably introduced with the glucose-1 phosphate) to initiate the reaction.

If the digests had been allowed to reach equilibrium it is probable that the only soluble polysaccharide produced would have been amylopectin, for the phosphate-independent action of Q-enzyme would have continued even after the establishment of the mineral phosphate–glucose-1 phosphate equilibrium by P-enzyme. Digestion was arrested therefore in each case when approximately 72% of the ester-phosphate had been converted into mineral phosphate, *i.e.*, shortly before phosphate equilibrium (75·5% conversion at pH 7·0; Hanes, *Proc. Roy. Soc.*, *B*, 1940, **129**, 174) was reached. As the proportion of active Q-enzyme was increased, the speed of the phosphate conversion also increased (Table IV). Thus, although the Q-enzyme preparation itself possessed negligible P-enzyme activity, it accelerated P-enzyme action by an autocatalytic effect (Part VI), which is shown also by Cori's " branching factor " (*J. Biol. Chem.*, 1943, **151**, 57). This marked autocatalysis can scarcely be attributed to the hydrolysing activity of amylase impurities in the Q-enzyme sample inasmuch as none of the digests showed a final reducing power (R_{Cu}) exceeding 2·7% in terms of conversion into maltose (Table IV).

From each of the first three digests the synthetic polysaccharide was obtained in two fractions, a water-insoluble fraction (Ia, IIa, and IIIa), separated by the centrifuge, and a soluble fraction (Ib, IIb, and IIIb), which was precipitated with alcohol from the supernatant liquid. In digests IV and V no insoluble component was formed. The total recovery of polysaccharide was always 90%, or more, of the amount estimated to be present on the basis of the final phosphate conversion (Tables I and V). Table I shows that the synthetic polysaccharides had very low copper-reducing powers and contained only traces of phosphate and other minerals.

When hydrolysed with acid, they gave high conversions into glucose, as estimated by cuprimetric titration. The optical rotatory powers of the polysaccharides in aqueous alkali were practically identical with those of potato starch and its components ($[\alpha]_D^{16}$ 156°). Qualitative tests indicated the absence of protein.

TABLE I.

Purity of the synthetic polysaccharides.

Poly-saccharide.	Recovery (%) based on phosphate conversion.	Ash (%).	$[\alpha]_D^{16}$ in 0·5N-NaOH.	Total phosphorus (%).	R_{Gl} in terms of glucose (%), before acid hydrolysis.	after acid hydrolysis.
Ia	69·6	0·46	157°	0·25	0·5	92
Ib	28·6	0·44	155	0·02	0·2	98
IIa	95·7	0·95	158	0·17	0·5	91
IIb	5·6	1·77	157	0·09	0·7	91
IIIa	41·3	1·13	161	0·10	0·4	95
IIIb	53·5	0·40	158	0·00	0·3	98
IV	89·9	0·86	160	0·07	0·4	99
V	96·8	0·45	160	0·00	0·2	95

TABLE II.

Characteristics of the synthetic polysaccharides.

Poly-saccharide.	Iodine complex. B.V.	Approx. λ of peak absorption (A.).	Activating power for P-enzyme (soluble starch = 1).	Limit conversion (%) into maltose with soya-bean β-amylase.	cryst. β-amylase.	Fraction (%) precipitated by thymol.
Potato amylose *	1·34	6500	0·37	—	—	—
I	(1·08)	—	(0·43)	(75)	(70)	(75)
Insol. Ia	1·18	6150	0·41	89	85	82
Sol. Ib	0·82	5800	0·49	41	35	58
II	(0·82)	—	(0·59)	(68)	(66)	(81)
Insol. IIa	0·84	5800	0·59	69	68	86
Sol. IIb	0·44	5600	0·62	46	40	0
III	(0·31)	—	(0·87)	(63)	(63)	(31)
Insol. IIIa	0·42	5700	0·79	68	66	71
Sol. IIIb	0·22	5500	0·94	60	60	0
IV	0·15	5300	0·97	59	58	0
V	0·12	5200	1·02	57	58	0
Potato amylopectin	0·18	5600	1·03	56	—	0

* From Part III (*J.*, 1949, 1448).

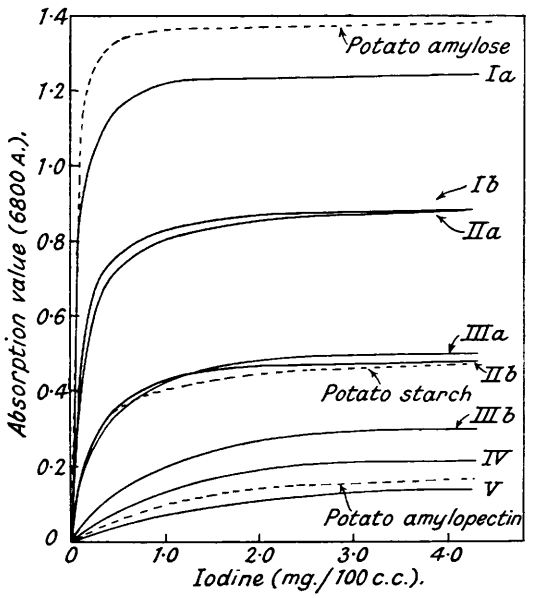
The effect of increasing the proportion of Q-enzyme is seen in better perspective if the properties of the " whole " polysaccharide produced in a given digest are considered rather than the properties of the soluble and the insoluble fractions separately. The values given in Table II for the polysaccharides, I, II, and III, have been calculated, as weighted averages based on yields, from the measured values for the respective soluble and insoluble fractions.

It is seen that there is a uniform gradation in the properties of the " whole " polysaccharides which is indicative of an increasing degree of branching as the proportion of Q-enzyme in the synthesising mixture is increased. Thus, with increasing Q/P ratio in the digests I—V it is found that (i) the blue values of the products vary from 1·08 to 0·12 and at the same time the wave-lengths of the absorption peaks move towards that of waxy maize starch (5200 A.; Part V, *J.*, 1949, 1712), (ii) the activating power of the polysaccharides with respect to P-enzyme synthesis, which is to some extent a measure of the number of chain-ends per unit weight, increases from 0·43 to 1·02 (soluble starch = 1·0), and (iii) the limit of conversion into maltose by soya β-amylase (which is inversely related to the number of branch points) varies from 75% (polysaccharide I) to 57% (polysaccharide V). Furthermore, while polysaccharides I and II are largely precipitated by thymol, less than a third of III shows this " amylose " characteristic and IV and V are not precipitated at all.

When, instead of the " whole " polysaccharides, the soluble and insoluble fractions of the first three are examined, the heterogeneity of the synthetic products is brought into prominence. Particular interest attaches to fraction Ib (and to a smaller extent to IIb) which displays certain anomalies of structure not found in any fraction separated hitherto from a natural starch. Although fraction Ib was soluble in the incubation solution, more than half of it was precipitable

as a thymol complex. Furthermore its blue value (0·82), the variation of A.V. (6800 A.) with increasing iodine–iodide concentration (see Fig.; cf. Baldwin, Bear, and Rundle, *J. Amer. Chem. Soc.*, 1944, **66**, 111) and its activating power in synthesis (0·49) suggest a structure of moderately long chains with a limited degree of branching. Nevertheless, fraction Ib was hydrolysed to the extent of only 41% by soya-bean β-amylase and 35% by crystalline sweet-potato β-amylase and thus appeared to be more highly branched than potato amylopectin. A possible explanation of this apparent anomaly is that fraction Ib is composed of fairly long chains having a certain degree of branching near their non-reducing ends. This view derives support from the observation that the limit dextrin produced by β-amylolysis from fraction Ib is stained blue, not red, with iodine. It is unlikely that digest I was entirely free from Q-enyzme activity and so polysaccharides Ib and IIb probably resulted from a very mild Q-enzyme action. If Q-enzyme, like P-enzyme and β-amylase, initially attacks non-reducing chain-ends, the early stages of Q-enzyme action on amylose may well be expected to give rise to a structure of the type postulated above for fraction Ib.

Polysaccharide–iodine colours produced with variation of iodine concentration.



Polysaccharide, 1 *mg.*/100 *c.c.*; *potassium iodide*, 10 × *iodine concentration*; *readings taken after* 24 *hrs.*).

The velocity constant of β-amylolysis of a starch polysaccharide is mainly determined by the number of non-reducing chain-ends available in unit weight, so that amylopectin is degraded more readily than is amylose (Part V). Yet further evidence that the higher the Q/P ratio in the synthesising digest the more extensive is the branching of the resulting polysaccharide was provided by a comparison of the rates of β-amylolysis of the precipitated fraction, Ia, and the soluble polysaccharide, IV. The reaction during the first 40 minutes was of the first order in both cases and the respective velocity constants obtained with identical enzyme concentrations were 4·25 and $7{\cdot}25 \times 10^{-2}$ (min.$^{-1}$). Clearly, β-amylolysis proceeds more readily with polysaccharide IV which must therefore present more chain-ends to the action of the enzyme than does fraction Ia.

There is thus no doubt that the mixtures of P- and Q-enzymes had synthesised a range of polysaccharides and that the extremes approximated in properties to natural amylose and amylopectin. Nevertheless, the synthetic polysaccharides cannot be regarded simply as mixtures of two molecular species, namely, of an unbranched long-chain amylose and a highly branched, short-chain amylopectin. On the contrary, the gradation in properties shown by the synthetic polysaccharides is to be ascribed rather to variations in molecular structure. These variations find expression in the average length of the basal chains synthesised by the P-enzyme component and in the number and mean position of the branch linkages introduced by Q-enzyme.

Further evidence regarding the structure of these synthetic polysaccharides has been

obtained by methylation and end-group assay. These results are reported in the following communication.

Experimental.

Dipotassium Glucose-1 Phosphate from Potato Starch.—The Cori ester was prepared from potato starch by phosphorolysis, as recommended by Sumner and Somers (*Arch. Biochem.*, 1944, **4**, 11). Thrice recrystallised, it showed $[\alpha]_D^{18}$ +77·2° (*c*, 3·73 in water) (Found: total P, 8·3; free P, nil. Calc. for $C_6H_{11}O_9PK_2,2H_2O$: total P, 8·3%).

Isolation of P-Enzyme from Potatoes.—A stock sample of fraction P2, which had been stored for several months as the free-flowing powder obtained when a solution of the enzyme was freeze-dried by the standard procedure reported in Part VI, was further purified by two fractional precipitations with neutral ammonium sulphate, the enzyme fraction being precipitated between salt concentrations of 19 and 35 g. per 100 c.c. being retained at each stage. The product was dissolved in 0·2M-citrate buffer (pH 6·0; 25 c.c per 100 c.c. of potato juice employed) and freeze-dried. When required for use in the digests, the enzyme was dissolved in water (50 c.c. per 100 c.c. of potato juice employed).

This solution possessed 3·1 units of phosphorylase per c.c. (determined by the method of Green and Stumpf, *J. Biol. Chem.*, 1942, **142**, 355).

Isolation of Q-Enzyme from Potatoes.—A stock freeze-dried sample of fraction Q3, prepared by the standard method (Part VI), was further purified by two precipitations with neutral ammonium sulphate (18 g./100 c.c.). The product was dissolved in 0·2M-citrate buffer (pH 6·5; 13 c.c. per 100 c.c. of potato juice employed) and freeze-dried. When required, the enzyme was dissolved in water (125 c.c. per 100 c.c. of potato juice employed), giving a solution which possessed $1{\cdot}1 \times 10^{-2}$ units (Green and Stumpf) of phosphorylase per c.c.

Synthesis of Polysaccharides from Glucose-1 Phosphate.—(a) *Conditions of synthesis.* Five digests, having the compositions shown in Table III, were prepared and incubated at 25°. The inactivated Q-enzyme solution was obtained by boiling a portion of the active solution, removing the coagulated protein by filtration, and diluting the filtrate to compensate for volume loss. At intervals aliquot portions of each digest were removed for the determination of (1) reducing power, by the method of Shaffer and Hartmann (*J. Biol. Chem.*, 1921, **45**, 377), and (2) mineral phosphate, by the colorimetric method of Allen (*Biochem. J.*, 1940, **34**, 858). Since the Shaffer–Hartmann reagent is insensitive to small amounts of reducing sugar (<0·2 mg. of glucose and <0·4 mg. of maltose), a known weight of maltose was introduced during the estimation and the reducing power of the test portion was calculated by difference.

Table III.

Composition of the digests.

Each digest contained glucose-1 phosphate (39·06 g.), 0·2M-citrate buffer (pH 6·3; 600 c.c.), and the enzyme solutions given below.

Digest number:	I.	II.	III.	IV.	V.
P-Enzyme solution (c.c.)	225	225	225	225	225
Active Q-enzyme solution (c.c.)	0	28	84	141	225
Inactivated Q-enzyme solution (c.c.)	225	197	141	84	0

The digests were incubated until approx. 72% of the Cori ester had been converted into polysaccharide, as shown by the phosphate estimations. The higher the Q-enzyme content, the more rapidly was this stage attained (see Table IV). The final pH was *ca.* 7·3, compared with 7·1 initially.

In those digests (IV and V) which were free from precipitate, the enzymes were inactivated by a few minutes' boiling, and the coagulated protein was removed by filtration.

In digests I, II, and III where an insoluble polysaccharide had been formed, each digest was centrifuged a short time before the requisite phosphate conversion was reached and the final phosphate estimation was made on the supernatant liquid, which was then immediately boiled and filtered to remove the coagulated protein. The insoluble polysaccharide was washed twice with water, the washings being boiled, filtered, and added to the main filtrate.

(b) *Treatment of the insoluble polysaccharide fractions.* Each insoluble polysaccharide was ground twice with 95% alcohol, once with absolute alcohol, and twice with ether, and dried at 60° in a vacuum over phosphoric oxide.

(c) *Treatment of the soluble polysaccharide fractions.* The filtrate obtained when the coagulated protein was removed was concentrated at 0° to 250 c.c. and dialysed against distilled water (frequently changed) for 36—48 hours. In no case did iodine-staining material penetrate the Cellophane membrane. The polysaccharide was precipitated with alcohol (2—3 volumes), coagulation being facilitated by the addition of a trace of sodium chloride. After 24 hours, the precipitate was removed by the centrifuge, and dried as above.

Tables IV and V record the phosphate conversions and reducing powers of the digests at the end of the various incubation periods, and the yields of the polysaccharide fractions.

Examination of the Synthetic Polysaccharides.—(a) *Ash content.* The polysaccharide (20 mg.) was heated in a micro-muffle until there was no further change in weight.

(b) *Tests for protein impurity.* The polysaccharide was subjected to Millon's test and the sodium fusion test for nitrogen.

TABLE IV.

Phosphate conversions and reducing powers of the digests.

Digest number.	Time of incubation (hours).	Final R_{Cu} (as " apparent % conversion into maltose ").	Final % conversion of ester-phosphorus into free-phosphorus.
I	47·5	0·6	71·5
II	39·5	1·0	72·1
III	29·0	1·4	68·7
IV	27·0	2·1	71·0
V	27·0	2·7	71·9

TABLE V.

Yields of synthetic polysaccharides.

	Precipitated fraction (*a*).		Soluble fraction (*b*).	
Digest number.	Weight (g.).	% yield based on phosphate conversion.*	Weight (g.).	% yield based on phosphate conversion.*
I	8·39	69·6	3·45	28·6
II	11·61	95·7	0·69	5·6
III	4·76	41·3	6·18	53·5
IV	Nil	—	10·74	89·9
V	Nil	—	11·56	96·8

* Allowance has been made for the aliquot portions of the digests removed for analysis.

(c) *Acid hydrolysis to glucose.* The polysaccharide was hydrolysed with 7% sulphuric acid at 100°, as described by Bourne, Donnison, Haworth, and Peat (*J.*, 1948, 1687). The amount of glucose formed was estimated by means of the Shaffer–Hartmann copper reagent.

(d) *Iodine stain.* A slightly acid solution of the polysaccharide was stained with iodine–potassium iodide under the standard conditions described by Bourne, Haworth, Macey, and Peat (*J.*, 1948, 924). In this way the blue value (B.V.) and absorption values (A.V.; λ. 4300—6800 A.) were measured. These terms were defined by Bourne, Haworth, Macey, and Peat (*loc. cit.*) and by Bourne, Donnison, Haworth, and Peat (*loc. cit.*).

In addition, the variation of A.V. (6800 A.) with iodine concentration was examined. For this purpose, a solution of the polysaccharide (1 mg.), prepared as above, was treated with increasing volumes of an iodine (0·2%)–potassium iodide (2·0%) solution in a final volume of 100 c.c.

(e) *Optical activity.* The polysaccharide was dissolved, with gentle warming, in 0·5N-sodium hydroxide, giving a 0·5% solution, which was used without neutralisation for optical activity measurements.

(f) *Priming activity in synthesis by phosphorylase.* For measuring the ability of the polysaccharide to act as a primer of the synthetic function of potato phosphorylase, the procedure reported in Part III (*J.*, 1949, 1448) was employed, except that a smaller amount (1 mg.) of polysaccharide was used. The activating power quoted in Table II is the ratio of the weight of mineral phosphate liberated from the Cori ester in the presence of 1 mg. of the polysaccharide to that liberated in the presence of 1 mg. of commercial soluble starch (AnalaR), allowance being made for the relatively small priming powers of any activators already present.

(g) *Limit of β-amylolysis.* A solution of the polysaccharide (20 mg.) in 0·1N-sodium hydroxide (25 c.c.) was exactly neutralised with 0·5N-hydrochloric acid and diluted to 34 c.c. After the addition of M-acetate buffer (pH 4·7; 6 c.c.) and 0·2% β-amylase solution (10 c.c.; prepared from soya beans by the method of Bourne, Macey, and Peat, *J.*, 1945, 882), the digest was incubated at 20° for a maximum of 6 hours. At intervals the reducing power was determined, in terms of maltose, by the Shaffer–Hartmann method (*loc. cit.*), a correction being applied for the small reducing power of the enzyme. The polysaccharides having a high B.V. showed a tendency to retrograde at pH 4·7 unless the β-amylase solution was added immediately.

In addition, the limiting conversion effected by crystalline β-amylase was ascertained. This enzyme preparation was used in a concentration equivalent to that of the soya-bean β-amylase.

(h) *Selective precipitation with thymol.* A solution of the polysaccharide (*ca.* 300 mg.) in 0·5N-sodium hydroxide (5 c.c.) was diluted to 100 c.c. and exactly neutralised with sulphuric acid. Powdered thymol (0·5 g.) was added and the mixture was kept at 30° for 36 hours.

The precipitate of polysaccharide–thymol complex was removed by the centrifuge and washed twice with saturated thymol-water. The complex was washed thoroughly with absolute alcohol and then with ether, and the residual polysaccharide was dried to constant weight in a vacuum over phosphoric oxide. The results are tabulated below.

The other synthetic polysaccharides gave no precipitate with thymol.

Treatment of the synthetic polysaccharides with thymol.

Polysaccharide	Ia	Ib	IIa	IIIa
Wt. taken (mg.)	300	280	374	380
Thymol-precipitated fraction (mg.)	245	162	320	271
,, ,, (%)	82	58	86	71

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