233. *The Enzymic Synthesis and Degradation of Starch. Part I. The Synthesis of Amylopectin.*

By E. J. Bourne and S. Peat.

The synthesis from glucose-1-phosphate of the unbranched amylose component of starch is catalysed by a phosphorylase found by Hanes in potato juice. We have now isolated from the same source an enzyme (Q-enzyme) which, acting in conjunction with the purified phosphorylase (P-enzyme), effects the conversion of glucose-1-phosphate into the major component of whole starch, namely amylopectin. The synthetic amylopectin is non-reducing, does not retrograde from solution, stains red-purple with iodine and is attacked by β-amylase in the manner characteristic of branched chain structures, *i.e.*, hydrolysis proceeds with the liberation of maltose until a limit represented by a conversion of 46% is attained. Thereafter the action of β-amylase ceases. In these properties, the synthetic product is not to be distinguished from natural amylopectin (separated from potato starch). Furthermore, methylation of the synthetic amylopectin and end-group assay on the methyl ether show the unit chain to contain 20 glucose members.

The probable course of the synthesis of whole starch in the plant is outlined and the respective roles of the P- and Q-enzymes therein are described. As an intermediate in the conversion of glucose-1-phosphate to amylose and amylopectin, the existence of a pseudo-amylose is postulated. Pseudo-amylose is constituted of an unbranched chain of 20 glucose units mutually linked by α-1 : 4-glucosidic bonds, as in amylose.

Two discoveries of the greatest significance in regard to the metabolism of carbohydrates in plants have been made in recent years. In the first place, it has been established beyond reasonable doubt that most natural starches are constituted of at least two different molecular species. The earlier names of "amylose" and "amylopectin," although originally connoting components of starch which differed only in the degree of physical aggregation of a common chain-unit, have been retained to describe these chemically distinct polyglucoses. Amylose is composed of long unbranched chains of glucose residues mutually combined by α-1 : 4-glycosidic links. Amylopectin, on the other hand, consists of comparatively short chains of glucose residues similarly linked, these chain units (containing an average of 20 glucose members) * being cross-linked by primary bonds which are very probably α-1 : 6-glycosidic links (cf. Barker, Hirst and Young, *Nature*, 1940, **147**, 296). This lateral linkage has the effect of giving to the amylopectin component of starch a highly branched or laminated structure, comparable to that of glycogen, whereas the amylose has a linear and probably unbranched structure similar to that of cellulose. The average length of the amylose chain in natural starch is 200—350 hexose members (Hassid and McCready, *J. Amer. Chem. Soc.*, 1943, **65**, 1157; K. H. Meyer, *et al.*, *Helv. Chim. Acta*, 1940, **23**, 66; 1941, **24**, 378).

The properties of amylose and amylopectin separated from a naturally occurring starch are very different. Two such properties intimately concern us since they are used for diagnostic and analytical purposes. These are (i) the different colours which the two components of starch give with iodine and (ii) the different hydrolytic effects which β-amylase exerts on each.

The second significant discovery, made by Hanes, was that of an enzyme found in a number of higher plants, which was capable of bringing about the synthesis from glucose-1-phosphate of a polyglucose with starch-like properties (Hanes, *Proc. Roy. Soc.*, 1940, B, **128**, 421; *ibid.*, **129**, 174).

This starch-like polysaccharide exhibited all the properties associated with the amylose, as distinct from the amylopectin component of natural starch. End-group assay on this 'synthetic starch' showed the chain length to be 80—90 glucose residues (Haworth, Heath and Peat, J., 1942, 55) and the determination of the molecular weight of its methyl ether by osmotic pressure measurement indicated the absence of branching (unpublished result).

The enzyme isolated by Hanes, and called a "phosphorylase," was extracted from potato juice and purified by a procedure designed to remove any starch-hydrolysing amylases the juice might contain. Of the total polysaccharide synthesised by the action of potato phosphorylase on glucose-1-phosphate, 85% separated from solution as "granular synthetic starch." The remainder (15%), which did not separate from solution although it gave a colour with iodine, was not further examined.

It was always evident that Hanes' phosphorylase represented only a part of the enzyme system involved in the biological synthesis of starch inasmuch as the greater part of the product formed by its agency consisted of the amylose component. Amylopectin which actually constitutes about 80% of natural potato starch appears not to be produced from glucose-1-phosphate by purified phosphorylase.

It was important therefore to examine the whole carbohydrase system of the potato with a view to determining what other factors in addition to purified phosphorylase (termed "P-enzyme" in what follows) were operative in the synthesis of whole starch in the potato. We instituted a search with this object in view and, as briefly reported elsewhere (Haworth, Peat and Bourne, *Nature*, 1944, **154**, 236), we have been able to show the presence in potato juice of another enzyme (Q-enzyme) which is so able to modify the normal function of P-enzyme that the combined action of the two leads to the formation not of amylose but of the branched-chain component, amylopectin.

The method adopted by Hanes for the elimination of amylases and the preparation of a pure phosphorylase consisted in the fractional precipitation of fresh potato juice by progressively increasing concentrations of ammonium sulphate solution. The first precipitated fraction, deemed to contain the amylases and to be

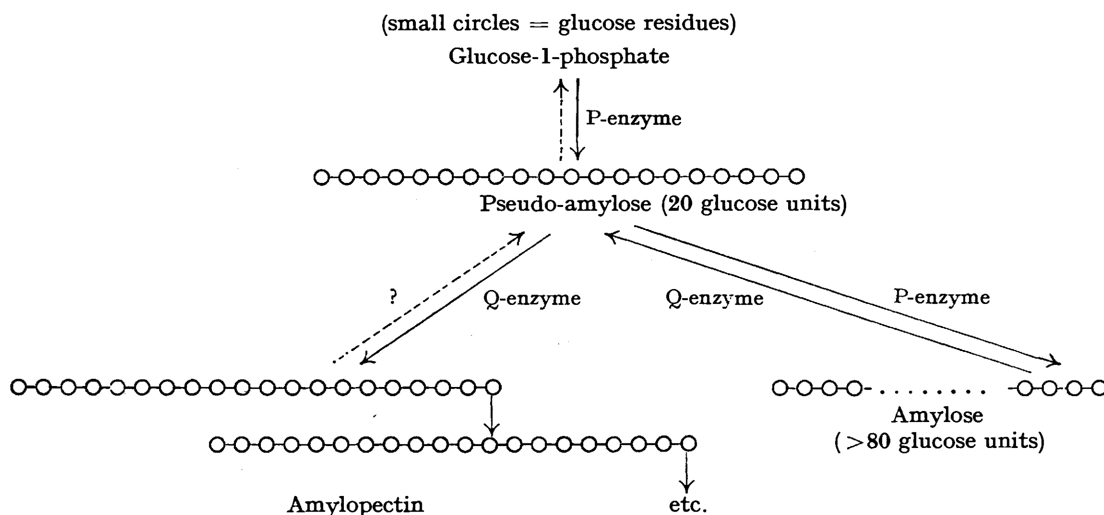
* Recent unpublished determinations indicate that the average unit chain-length of potato amylopectin is more nearly represented by 20 than by 24 glucose numbers.

substantially free from P-enzyme, was discarded by Hanes; it was in this fraction that we detected the presence of Q-enzyme.

Although our study of the properties and function of Q-enzyme have opened up new lines of investigation which we are still pursuing, our investigations have progressed sufficiently to warrant the publication of this account. The picture now presented of the mechanism of starch synthesis is to be regarded as a working hypothesis only.

The synthesis of polysaccharide from glucose-1-phosphate is a reversible reaction and Hanes has shown that the equilibrium is determined by the concentrations of the bivalent ions, HPO_4'' and $(C_6H_{11}O_5{\cdot}O{\cdot}PO_3)''$, the ratio of which is constant (2·2) at any pH. The phosphorolysis of starch by P-enzyme is dependent upon the concentration of inorganic phosphate but is independent of the concentration of starch.

We regard the function of P-enzyme as being restricted to the formation of α-1 : 4-glycosidic linkages by the union of molecules of glucose-1-phosphate and liberation of phosphate ions and to the reverse process of scission of these glycosidic linkages in the presence of inorganic phosphate. Unbranched chains of glucose residues are thus produced by the end-wise apposition of glucose residues derived from glucose phosphate. This being so, it is seen that pure P-enzyme cannot catalyse the synthesis of branched chains and some other agent must be influential in the synthesis of amylopectin. It is this function, namely, the formation of 1 : 6-glucosidic cross linkages between chains of glucose units, themselves linked by 1 : 4-linkages, that we ascribe to Q-enzyme. The picture we have conceived of the mechanism of starch synthesis is this : P-enzyme acting on glucose phosphate forms chains of glucose units with the production, in the absence of Q-enzyme or when the proportion of Q-enzyme is below a critical value, of amylose. If the proportion of Q- to P-enzyme is above this critical value, the normal amylose synthesis is interrupted inasmuch as the building up of linear chains by P-enzyme ceases when the average length of these chains attains about 20 glucose units. Chains of this average length (we use the term " pseudo-amylose " to describe this hypothetical polysaccharide) constitute the only suitable substrate for the synthetic activity of Q-enzyme which then proceeds to link them together, in the manner already indicated, to give the laminated, amylopectin structure. The synthesis of amylose and amylopectin by the agency of P- and Q-enzymes is illustrated diagrammatically thus :



The steps in the reversal of this synthesis are less easy to define. In our use of the terms P- and Q-enzymes we are referring to the pure enzymes, *i.e.*, to preparations which contain no other carbohydrase than that specified as P or Q. This state of purity has certainly not yet been attained with Q-enzyme and it is indeed doubtful whether P- has been obtained entirely free from other enzymes. In large scale preparations of glucose-1-phosphate it is customary to use crude potato juice as the enzyme source and by its agency both of the components of whole starch are converted into glucose phosphate. This observation is based not upon the actual yield of glucose phosphate but upon estimation of the extent of conversion of inorganic phosphate to ester-phosphate.

Hanes (*loc. cit.*) states that the same total conversion of starch into glucose phosphate is effected by the " purified phosphorylase " as by crude juice. Since we have reason to believe from our own experiments that this enzyme preparation may contain small proportions of Q-enzyme, it cannot be asserted at the present time that Q-enzyme is not a necessary ancillary agent in the phosphorolysis of whole starch or that P-enzyme unmixed with Q-enzyme is capable of phosphorolysing starch.

We have been able to show that Q-enzyme exerts not only the synthesising activity illustrated above, but also possesses an amylose-hydrolysing function which is different from the normal course of hydrolysis brought about by the known amylases. An account is published in Part II of the amylolytic activity of Q-enzyme and it will be sufficient to mention here that Q-enzyme, when free from α- and β-amylases, converts

blue-staining amylose into a red-staining polysaccharide probably without the concomitant liberation of any reducing sugar during the whole course of the digestion. This is in marked contrast to the action on amylose of salivary α-amylase and of soya bean β-amylase; the former rapidly converts amylose to non-staining dextrins and maltose, and the latter liberates maltose from the beginning of the reaction with ultimate complete saccharification of the amylose. Our method of estimating reducing power was adequate to reveal the formation of feebly reducing dextrins and, as these were apparently absent, we incline to the view that Q-enzyme actually catalyses the conversion of amylose into amylopectin, possibly through the intermediate formation of pseudoamylose, as indicated diagrammatically above. Of the action of Q-enzyme on amylopectin we are at present ignorant, but it may be that the reaction: pseudo-amylose ⟶ amylopectin, is reversible. We do, at least, know that amylopectin is convertible into glucose-1-phosphate, although the intermediate steps in this reaction have not yet been determined.

The Synthesis of Amylopectin from Glucose-1-phosphate.—Potato juice often contains amylases, *i.e.*, enzymes which effect the hydrolysis of starch with the liberation of reducing groups. The presence or absence of amylases appears to depend on the stage of growth of the potato; they are particularly in evidence in sprouting potatoes. The action of Q-enzyme on starch bears a superficial resemblance to the action of the true amylases (as is shown in Part II) and it was therefore essential that preparations of Q-enzyme should be freed as far as possible from amylases. This was in part achieved by the potato juice being shaken with kaolin under the conditions described by Waldschmidt-Leitz and Mayer (*Z. physiol. Chem.*, 1935, **236**, 168). Thereafter the Q-enzyme was precipitated by neutral ammonium sulphate at a final concentration of 16 g./100 c.c. P-enzyme was prepared from fresh potato juice by Hanes' method, which involves the use of ammonium sulphate at a concentration of 35 g./100 c.c. Q- and P-enzymes were purified by repeated precipitation at the appropriate concentration of ammonium sulphate.

In a qualitative study of the action of P-, of Q- and of mixtures of P- and Q-enzymes on glucose-1-phosphate it was observed that whereas P-enzyme rapidly gave a blue-staining polysaccharide without the intermediate formation of a red-staining product, the P + Q-mixture gave a red-staining polysaccharide. The latter was also given, but very much more slowly, by our earlier preparations of Q-enzyme. This synthetic activity of Q-preparations diminished as the purity of Q- increased and we believe that a Q-preparation entirely free from P-enzyme will show no synthetic activity with respect to glucose phosphate as substrate. In a further experiment it was shown that if a Q-preparation was (*a*) submitted to a temperature of 65° for 15 minutes or (*b*) precipitated from aqueous solution with alcohol, it entirely lost its characteristic property of influencing the course of polysaccharide synthesis by P-enzyme.

In a larger scale synthesis of the red-staining polysaccharide, it was not convenient to prepare and purify separately the P- and Q-factors. Instead, potato juice which had been submitted to the kaolin treatment to remove amylases was used as the source of P + Q-mixtures. The relative proportion of P- and Q-enzymes would be expected to exert a predominating influence on the type (amylose or amylopectin or mixtures thereof) of polysaccharide synthesised, and it is to be remembered that the kaolin-treated juice most probably does not contain the same P/Q ratio as the fresh untreated juice. It is known for instance, that both P- and Q-enzymes are adsorbed by kaolin although not to the extent of the amylases.

The polysaccharide formed by the action of kaolin-treated potato juice on glucose-1-phosphate was shown to have the constitution and properties of amylopectin. It was easily soluble in water; it stained red with iodine and it was hydrolysed by β-amylase in a manner identical with the course of hydrolysis of natural amylopectin by the same enzyme. The polysaccharide synthesised by P-enzyme acting alone was completely hydrolysed (99%) to maltose by β-amylase, whereas the product of synthesis by P + Q-enzymes was converted into maltose to the extent of 46% only. A natural amylopectin, prepared from potato starch by the butanol method of Schoch (*J. Amer. Chem. Soc.*, 1942, **64**, 2957), was converted into maltose to the extent of 50% by the same amylase preparation.

The branched chain structure for the synthetic amylopectin was confirmed when end-group assay on the methyl derivative showed that its unit chain length was approximately 20 glucose members corresponding to the unit chain length of natural amylopectin (K. H. Meyer, *Helv. Chim. Acta*, 1940, **23**, 865; Hassid and McCready, *J. Amer. Chem. Soc.*, 1943, **65**, 1157). Viscosity measurements on chloroform solutions of the methylated polysaccharide showed that $\eta_{sp.}/c$ * was independent of c, a property which distinguishes the branched from the unbranched structure. In the latter case, $\eta_{sp.}/c$ increases with c.

EXPERIMENTAL.

Preparation of Potato Juice.—King Edward potatoes (1000 g.) were peeled, thinly sliced and soaked for 30 minutes in water (1 litre) containing 0·5% sodium hydrosulphite (dithionite) and 0·5% toluene. They were drained, washed, minced and pressed. The light yellow juice was clarified by centrifuging and, after addition of 0·5% of toluene, was stored at 0°.

Measurement of Synthetic Activity (Qualitative).—A digest consisting of 0·1 c.c. enzyme solution, 0·2 c.c. 0·1M glucose-1-phosphate and 0·1 c.c. citrate buffer (pH = 6·0) was incubated at 25° for 15 minutes. Three drops of an iodine-potassium iodide solution (N/60) were added. The production of a coloured starch-iodine complex indicated the presence of phosphorylase in the enzyme.

Purification of Phosphorylase (P-Enzyme).—A solution of ammonium sulphate containing 50 g./100 c.c. was prepared by dissolving ammonium sulphate in water and adding ammonium hydroxide (d = 0·880) until the pH was 7·0. Small amounts of insoluble impurity were removed by filtration and the filtered solution was saturated with toluene.

* The specific viscosity, $\eta_{sp.}$, of a solution is defined by the expression, $\eta_{sp.} = \eta_r - 1$, where η_r is the ratio of efflux times (in an Ostwald viscometer) of solution and solvent respectively; the concentration, c, is in grams/litre.

Potato juice was fractionated in the following manner, the temperature being maintained at 0° throughout. To each 100 c.c. of juice, ammonium sulphate solution (47 c.c.) was added giving a concentration of 16 g./100 c.c. The precipitate was removed on the centrifuge and discarded. To the supernatant liquid more ammonium sulphate solution (180 c.c.) was added, making the concentration 35 g./100 c.c. After standing for 4 hours, the phosphorylase precipitate was removed by centrifuging, and the supernatant liquid was discarded. The precipitate was redissolved in the minimum volume of water and refractionated twice. The thrice-precipitated phosphorylase thus isolated was termed "P-enzyme."

Estimation of phosphorylase activity was carried out by the method of Green and Stumpf (*J. Biol. Chem.*, 1942, **142**, 355), the inorganic phosphorus being estimated by the method of Allen (*Biochem. J.*, 1940, 858).

Polysaccharide Synthesis by P-Enzyme.—The P-enzyme used had been stored as a dry powder at 0° for 6 weeks and contained a considerable proportion of ammonium sulphate. The inorganic phosphorus in a digest of glucose-1-phosphate with P-enzyme was estimated at intervals by the method described and the results are given below.

25·0 G. glucose-1-phosphate, 1·0 g. dried P-enzyme (containing ammonium sulphate), 10 c.c. toluene, made up to 2 litres, pH maintained at 6·0 with acetic acid, temp. 20·0°.

Incubation time (hrs.).	Inorganic phosphorus in digest (g.).	Conversion (%).	Incubation time (hrs.).	Inorganic phosphorus in digest (g.).	Conversion (%).
0	0·008	0	70	1·240	60
24	0·412	20	120	1·680	81
46	0·788	38	168	1·728	83

When equilibrium at 83% conversion had been reached, the "granular synthetic starch" was separated by the centrifuge, washed with water, alcohol and ether and dried under reduced pressure over phosphorus pentoxide; yield, 5·75 g.

The addition to the supernatant liquid of iodine (2 g.) and potassium iodide (3 g.) in water (200 c.c.) caused the precipitation of more polysaccharide in the form of its iodine complex. This was removed by the centrifuge and washed with water. The iodine was removed by being repeatedly refluxed with acetone. The polysaccharide was dried under reduced pressure over phosphorus pentoxide and was termed "Soluble Synthetic Starch"; yield, 1·47 g.

Isolation of Q-Enzyme.—Potato juice was fractionated as described at 0° throughout. Potato juice (76 c.c.) was shaken for 15 minutes with 20 c.c. of buffer (pH = 5·1) and kaolin (5·4 g.) suspended in water (32 c.c.). The mixture was centrifuged and the kaolin, which should have retained any amylase which may have been present, was discarded.

To the supernatant liquid ammonium sulphate solution (63 c.c.) was added, the concentration of this salt in the mixture being thus 16 g./100 c.c. The resulting precipitate was removed by the centrifuge, redissolved in water (40 c.c.) and reprecipitated by the addition of ammonium sulphate (20 c.c.).

It was observed that Q-enzyme converted the starch into a reddish-purple staining polysaccharide in 24 hours. It was still reddish-purple staining after 9 days.

10 C.c. 1% potato starch solution + 1 c.c. Q-enzyme solution.

The Q-enzyme solution was prepared by dissolving the Q-enzyme precipitate in the minimum volume of water.

Time after mixing (mins.).	$\eta_r^{38°}$.	Iodine stain.	Time after mixing (days).	$\eta_r^{38°}$.	Iodine stain.
2	2·09	Blue	1	—	Red-purple
8	1·74	,,	5	—	,,
11	1·69	,,	9	—	,,
20	1·60	,,			
25	1·57	,,			

The Rôle of Q-Enzyme in Starch Synthesis.—The P-enzyme prepared from 100 c.c. of potato juice was redissolved in water (60 c.c.). The Q-enzyme prepared from 150 c.c. of juice was dissolved in water (30 c.c.). These two enzyme solutions were then used in the following digests, which were incubated at 38°. At intervals, two drops of each digest were removed, diluted with water (0·25 c.c.) and stained with one drop of N/60 iodine solution.

Iodine Stains.

5 c.c. 0·5M-Glucose-1-phosphate + 2 c.c. citrate buffer (pH = 6·0) +	0 min.	1 hr.	2 hrs.	24 hrs.	2 days.	5 days.	6 days.	7 days.	10 days.
1 c.c. P-enzyme	C	B(F)	B	B	B	B	B	B	B
1 c.c. Q-enzyme	C	C	C	RP	RP	R	R	R	R
1 c.c. P-enzyme + 1 c.c. Q-enzyme	C	R(F)	RP	RP	R	R	R	R	R

Key: B = blue, R = red, P = purple, (F) = faint, C = no stain.

Similar results were obtained on two other occasions using different preparations of P- and Q-enzymes.

The Destruction of Q-Enzymes by Heat or Alcohol.—P-Enzyme, from 300 c.c. of potato juice, was dissolved in water (100 c.c.) and the Q-enzyme from 300 c.c. of juice was dissolved in water (80 c.c.). The Q-enzyme solution was divided into three parts which were treated as follows: (i) centrifuged and the small insoluble residue discarded; (ii) heated at 65° for 15 mins., the coagulated protein being separated on the centrifuge and discarded; (iii) precipitated with three volumes of absolute alcohol. After being centrifuged, the precipitate was redissolved in one volume of water, the small insoluble residue being discarded. The following digests were then prepared and incubated at 25°. At intervals the iodine stains were examined.

(A): 5 c.c. citrate buffer (pH = 6·0) containing glucose-1-phosphate (0·05*M*), 0·5 c.c. P-enzyme solution, 6 c.c. Q-enzyme solution, layer of toluene.
(B): as (A), using heated Q-enzyme.
(C): as (A), using alcohol-precipitated Q-enzyme.

Iodine Stains.

Digest.	Incubation times.			
	0 min.	30 mins.	3½ hours.	5½ hours.
(A)	C	C	RP	RP
(B)	C	C	B	B
(C)	C	C	B	B

Key: R = red, B = blue, P = purple, C = no stain.

When the above experiment was repeated, using the same and other enzyme preparations, similar results were observed.

Syntheses using Q-Enzyme.—General method of isolation of the polysaccharide. The appropriate digest was prepared, covered with a layer of toluene and incubated at 25°. The inorganic phosphorus was estimated at intervals by the method of Allen (*loc. cit.*). When equilibrium was reached, the digest was boiled for 10 minutes and the coagulated protein was centrifuged off, washed with a little hot water and discarded. To the supernatant liquid and washings, methanol (3 vols.) was added and, after being kept for a short time, the polysaccharide precipitate was separated at the centrifuge.

It was redissolved in boiling water (200 c.c.), the solution filtered and dialysed for 5 days against running tap-water. Again methyl alcohol (3 vols.) was added and the polysaccharide was separated by centrifuging. After a further precipitation, the polysaccharide was washed with alcohol and ether and dried in a vacuum desiccator.

1. *By Q-enzyme acting alone. Polysaccharide RS*3.

Digest: 5·00 g. glucose-1-phosphate (K_2 salt), 20 c.c. citrate buffer (pH = 6·0), 20 c.c. water, 12 c.c. Q-enzyme solution. As the incubation proceeded three 12 c.c. volumes of fresh Q-enzyme solution were added. Equilibrium was reached in 10 days, 81% of the ester-phosphorus being converted into inorganic phosphorus; yield of polysaccharide RS3, 1·21 g.

2. *By mixtures of P- and Q-enzymes. Polysaccharide RS*4.

Digest: 5·00 g. glucose-1-phosphate (K_2 salt), 20 c.c. citrate buffer (pH = 6·0), 20 c.c. P-enzyme solution, 40 c.c. Q-enzyme solution, 20 c.c. water.

Equilibrium was reached in 3 days, 81% of the ester-phosphorus being converted into inorganic phosphorus; yield of polysaccharide RS4, 1·15 g.

3. *By untreated potato juice. Polysaccharide RS*5.

Digest: 5·00 g. glucose-1-phosphate (K_2 salt), 20 c.c. citrate buffer, 20 c.c. crude potato juice, 60 c.c. water.

Equilibrium was reached in 6 hours, 83% of the ester-phosphorus being converted into inorganic phosphorus; yield of polysaccharide RS5, 0·45 g.

Quantitative Estimation of the Effect of β-Amylase on the Synthetic Polysaccharides (with A. MACEY).

The standard digest, which was incubated at 35·5°, was: 30 mg. polysaccharide, 34 c.c. distilled water,* 6 c.c. acetate buffer (pH = 4·8), 10 c.c. 0·2% β-amylase prepared from wheat (see Ford, Haworth and Peat, J., 1940, 856), layer of toluene.

At frequent intervals, the amount of maltose formed was estimated by removing 5 c.c. of digest for analysis by the Shaffer–Hartmann method. The results are recorded below.

Hydrolysis of Polysaccharides by β-Amylase. % Conversion to Maltose.

Polysaccharide.	Initial solvent.		$\frac{1}{12}$ hr.	$\frac{1}{2}$ hr.	$1\frac{1}{2}$ hrs.	$2\frac{1}{2}$ hrs.	$3\frac{1}{2}$ hrs.	$4\frac{1}{2}$ hrs.	$5\frac{1}{2}$ hrs.	21 hrs.
Amylopectin (Butanol)	H_2O	(1)	—	26·0	41·5	47·6	—	49·4	50·4	—
(B.V., 0·18)		(2)	—	24·1	43·2	47·6	—	—	51·7	—
		(3)	—	25·3	39·6	45·2	45 5	46·7	46·7	—
Polysaccharide RS3	NaOH	(1)	13·5	39·8	57·0	65·5	65·0	65·5	65·5	—
(B.V., 0·19)		(2)	11·8	37·8	56·3	59·8	62·0	62·0	61·3	—
Polysaccharide RS4	H_2O	(1)	—	28·4	41·4	43·7	44·1	46·0	—	—
(B.V., 0·09)		(2)	—	28·2	39·5	42·8	43·7	43·7	45·5	—
"Granular Synthetic Starch"	NaOH	(1)	—	51·2	88·0	93·0	—	94·0	100·0	99·0
(B.V., 0·95)		(2)	—	55·5	93·0	98·7	—	98·5	98·7	98·5
			$\frac{1}{2}$ hr.	2 hrs.	4 hrs.	6 hrs.	8 hrs.	11 hrs.	$22\frac{1}{2}$ hrs.	28 hrs.
Amylose (Butanol)	NaOH	(1)	—	35·6	52·7	63·5	73·5	79·5	90·5	99·6
(B.V., 1·06)		(2)	—	39·2	—	63·4	72·7	79·7	91·0	98·0

Notes on Table: (1) "Butanol amylopectin" and "butanol amylose": these names refer to amylose and amylopectin separated from potato starch by fractionation with butanol (Schoch, *loc. cit.*).

(2) Blue value (B.V.) is a measure of the intensity of the blue in the colour of the polysaccharide–iodine complex formed under the standard conditions prescribed by McCready and Hassid (*J. Amer. Chem. Soc.*, 1943, **65**, 1154). The readings were made on a Spekker absorptiometer with a red filter.

(3) The B.V. of butanol amylopectin is higher than that of the polysaccharide RS4, the reason being that the butanol amylopectin still contains a little amylose. An alternative method of separation of the components of starch has been developed since the completion of this part of the work. In this method (an account of which will be published later) thymol and *cyclo*hexanol are used instead of butanol as precipitants and by its use amylopectin preparations with blue values as low as 0·04 have been obtained from potato starch.

Acid Hydrolysis of the Polysaccharides.—The polysaccharides listed in the last table were shown to be essentially pure polyglucoses by the fact that acid hydrolysis converted each into glucose to the extent of 98—100%.

The Amylopectin Synthesised by Kaolin-treated Potato Juice.—Isolation (with K. H. FANTES). The method used was that given above for the synthesis of polysaccharides. Instead of preparing the P- and Q-enzymes separately, potato juice was extracted with kaolin and then precipitated at an ammonium sulphate concentration of 35 g./100 c.c. The precipitate, containing P- and Q-enzymes, was dissolved in the minimum volume of water, for use in the digests. In seven digests, 16·66 g. of polysaccharide were isolated from 70·0 g. of glucose-1-phosphate, the recovery being 68% of the polysaccharide formed. All the digests were non-reducing, or only very slightly reducing, to Fehling's solution.

Methylation. The polysaccharide (12·6 g.) was dissolved in 4% sodium hydroxide solution (225 c.c.) and methylated at room temperature by the addition of methyl sulphate (150 c.c.) and 30% sodium hydroxide solution (375 c.c.), simultaneously and with vigorous stirring over a period of $2\frac{1}{2}$ hours. The mixture was then nearly neutralised with sulphuric acid and completely neutralised with carbon dioxide. After boiling for 30 minutes, the insoluble polysaccharide material was collected on a linen filter.

From the filtrate, sodium sulphate and sodium bicarbonate were removed by adding an equal volume of methanol and cooling to 0°. The crystals were separated and recrystallised from 50% methanol. The combined mother liquors were evaporated to dryness under reduced pressure. The residue and the partly methylated polysaccharide were dissolved

* When the polysaccharide could not be completely dissolved in warm water alone it was suspended in N-NaOH (25 c.c.) and solution was effected by gentle warming on a water-bath. The alkali was exactly neutralised with 5N-HCl, the solution was diluted to 34 c.c. and the rest of the digest was added as before.

in acetone (50 c.c.) and the methylation continued with 30% sodium hydroxide (400 c.c.) and methyl sulphate (150 c.c.) at 50°. Subsequent methylation treatments were carried out in the same way.

After the seventh methylation, the product, which was still somewhat sticky, was extracted from the linen cloth by being refluxed with eight successive volumes (200 c.c. each) of acetone. After being filtered the acetone extracts were combined and evaporated to a syrup which was twice extracted with hot ether (75 c.c.) to remove any acetone condensation products. A chloroform solution of the product was washed twice with water, dried with anhydrous sodium carbonate, filtered and evaporated to 75 c.c. Ligroin was added until a slight cloudiness was apparent. This was clarified by centrifuging. A large excess of ligroin was added to precipitate the methylated product which was separated on the centrifuge and hardened with ligroin. It was dried under reduced pressure and ground to a fine powder; yield, 7·0 g. (Found: OMe, 41·0; Ash, 0·4%); $[\alpha]_D^{18}$ +201° (*c*, 1·0 in chloroform). Measurement of viscosity of the methylated polysaccharide in chloroform showed $\eta_{sp.}/c$ was independent of c and = 0·016. This behaviour is typical of amylopectin derivatives and distinguishes them from the corresponding derivatives of amylose, with which $\eta_{sp.}/c$ increases as c is increased.

End-Group Assay.—Methanolysis. Dry methylated RS4 (5·806 g.) was dissolved in dry methanol containing 2·6% dry hydrogen chloride, making a 2·7% solution of the methylated polysaccharide. After being kept overnight, the solution was refluxed for 7½ hours. The hydrogen chloride was neutralised by being kept in contact for 20 hours with silver carbonate. The filtered solution was evaporated under diminished pressure. The last traces of silver salts were removed by dissolving the syrup in absolute ether, filtering and evaporating the solvent. The product was dried at 100°/15 mm.; yield 6·60 g.

Fractional distillation. The product was transferred, by means of ether, to a small distilling flask, containing glass wool and a little barium carbonate. After drying at 100°/15 mm. for ½ hour, it was distilled at bath temperature 150—170°/0·06 mm. into a tared Widmer flask, also containing glass wool. Distillate weighed 5·45 g. The syrup was then fractionally distilled from the Widmer flask, as shown below.

Fraction.	Bath temp.	Pressure (mm.).	Wt. (g).	n_D observed. Temp.	n_D observed. Reading.	$n_D^{16°}$ (calculated).	% OMe.	Wt. of "tetra-glucoside" (g.).
1	140—146°	0·018	0·096	22°	1·4420	1·4444	62·0	0·096
2	146—148	0·008	0·174	24	1·4462	1·4494	—	0·115
3	148—150	0·008	0·115	25	1·4470	1·4506	—	0·067
4	154	0·012	0·459	26	1·4526	1·4566	—	0·080
5	160	0·020	—	26	1·4552	1·4592	—	0·000
							Total =	0·358

The temperature coefficients used in the calculations for the table were those of Hirst and Young (J., 1938, 1247).

Fraction 1 was shown to be pure 2 : 3 : 4 : 6-tetramethyl methylglucoside, both by analysis (Theory: –OMe, 62·0%) and by comparison with the n_D value given by Hirst and Young ($n_D^{16°}$ 1·4445) for material which had been subjected to methanolysis under similar conditions. Fraction 5 was assumed to be pure 2 : 3 : 6-trimethyl methylglucoside. Using these two fractions as standards, the percentage of 2 : 3 : 4 : 6-tetramethyl methylglucoside in the other fractions was calculated. In all, 0·358 g. of this material was present. This corresponded to a chain-length of *20 units.*

This is necessarily only an approximate value because of (1) the incomplete methylation, (2) the omission to correlate $[\alpha]_D$ with n_D and (3) the small amount of material used for the end-group assay.

The authors wish to pay tribute to Professor W. N. Haworth, F.R.S., the Director of the Laboratories, for his encouragement during the course of this work and for most valued advice and criticism. They desire also to thank Messrs. A. Macey and K. H. Fantes for their assistance in part of the experimental work.

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[*Received, August 25th,* 1945.]