310. *The Enzymic Synthesis and Degradation of Starch. Part III. The Role of Carbohydrate Activators.*

By E. J. Bourne, D. A. Sitch, and S. Peat.

This paper is concerned with the function of carbohydrate activators in the synthesis of amylose by purified plant phosphorylase (P-enzyme). It lends support to the theory of Cori which pictures the synthesis as the growth of chain units from end-groups provided by the activator.

The activating powers of amylose and amylopectin have been compared, and the effects of (*a*) acid hydrolysis, (*b*) β-amylolysis, and (*c*) phosphorolysis on the activating power of each of these components of starch have been examined.

The conclusion is reached that potato P-enzyme functions by the endwise apposition of glucose residues (derived from glucose-1 phosphate) to a pre-existent chain (a "template chain") of 1 : 4-linked glucose members. The template chain may be an independent chain as in amylose, or an "outer" chain of a branched structure of the amylopectin type. It is shown that a template chain may consist of as few as 5—6 glucose members. Other workers, notably Weibull and Tiselius, have suggested that the minimum length of a template chain is three glucose units. Some evidence is also provided here that a template chain has an optimum, as well as a minimum length. This optimum length appears to be of the order of 20 glucose units.

The cross linkages of amylopectin are shown to constitute obstructions to the degradative function of P-enzyme as well as of β-amylase, and the striking inference can be drawn from this and previous work that none of the three enzymes examined, namely plant phosphorylase and the α- and β-amylases, is capable of inducing scission of the 1 : 6-glycosidic links of branched amylopectin structures.

Abundant evidence is now available that the polysaccharide-synthesising enzymes of both animal and plant tissues only utilise glucose-1 phosphate (the Cori ester) as substrate if a pre-formed polysaccharide of the starch type is also present, and the original observation of Cori and Cori (*J. Biol. Chem.*, 1939, **131**, 398) on muscle phosphorylase, and of Hanes (*Proc. Roy. Soc.*, 1940, *B*, **129**, 174) on potato phosphorylase (P-enzyme), have been fully substantiated by later investigations.

The work described in this paper makes a contribution to knowledge of the nature and function of such carbohydrate activators, and lends support to the hypothesis propounded by Cori *et al.* in 1943 (*J. Biol. Chem.*, **151**, 39). This hypothesis pictures the activator as providing end-groups which act as nuclei for the addition of new chain units, and was first proposed to explain the action of muscle phosphorylase but was later shown to be applicable also to potato phosphorylase. In a recent paper, Swanson and Cori (*J. Biol. Chem.*, 1948, **172**, 815) have presented more detailed evidence based on the activating power of the hydrolytic products of

branched and unbranched polysaccharides with respect to the phosphorylases of muscle and of potato.

Our own approach to the problem has been along similar lines, but has been confined to the plant phosphorylase (P-enzyme). Our technique has been somewhat different from that of Cori, and the results are in some points interpreted differently : in essentials, however, our observations are in agreement with his.

Methods.—The power of a number of polysaccharides to " activate " the P-enzyme synthesis of amylose from glucose-1 phosphate was measured by a modification of the method of Green and Stumpf (*J. Biol. Chem.*, 1942, **142**, 355) which is based upon a determination of the amount of inorganic phosphate liberated in unit time by the action of P-enzyme on purified potassium glucose-1 phosphate. This compound was prepared from starch by the biological method. The activating power of a polysaccharide is given not in absolute units but relative to that of a standard. The standard chosen was a commercial soluble starch. With each P-enzyme preparation a control is carried out using 5 mg. of this soluble starch as primer, and the activating power of the polysaccharide (5 mg.) under test is given in terms of the activating power of soluble starch as unity. By this means it was possible to correlate the results with P-enzyme preparations of different activity.

The P-enzyme was prepared from potato juice and was purified by repeated precipitation with neutral ammonium sulphate solution (35%). It was free from Q-enzyme, amylases, and maltase.

The amylose and amylopectin specimens were prepared by one of the methods of fractionation described by Peat *et al.* in earlier papers. As criterion of degree of separation of the components of starch we take the " blue value " (for definition see experimental section) of the polysaccharide–iodine complex. The purest amylose prepared from potato starch has a blue value of 1·45, the purest amylopectin fraction from this starch having B.V., 0·15. Starch fractions of intermediate blue values are to be regarded as mixtures of these two components, and it is thus possible to calculate the proportion of each in a fraction of known intermediate B.V. The blue values and activating powers of a number of starch polysaccharides are given in Table 1.

TABLE I.

Activator in P-synthesis (5 mg. per 3·5 c.c. digest).	B.V. of activator.	Activating power relative to soluble starch.
Soluble starch (standard)	0·41	1·0
Potato starch	0·47	0·88
Amylose (aluminium hydroxide)	1·34	0·37
Amylose (thymol)	1·18	0·53
Amylopectin (thymol)	0·21	0·95
Amylopectin (*cyclo*hexanol–MeOH)	0·51	1·06
Amylopectin (*cyclo*hexanol)	0·18	1·02
Amylopectin treated with salivary amylase	0·09	1·28
Dextrin-A (from thymol-amylopectin)	0·19	0·10
Dextrin-A (from whole starch)	0·15	0·08
Dextrin-P (by P-enzyme on amylopectin)	0·19	0·65
Synthetic amylose I (maximum chain length)	1·33	0·23
Synthetic amylose II	0·40	1·05
Synthetic amylose III	0·27	1·19
Maltose	—	0

It is obvious from the results given in Table I that the two components of starch are markedly different with respect to their efficacy as activators in the synthesis of amylose by P-enzyme. At the concentrations employed, amylopectin is 2—3 times as active as amylose. Indeed, the purest sample of undegraded amylopectin (B.V., 0·15) is 2·9 times as active as the purest sample of amylose used (B.V., 1·34).

The higher the blue value of an amylose preparation, the smaller is its content of the branched-chain component, and it can be said with assurance that an amylose of B.V. 1·34 contains only a small proportion of amylopectin. It must be concluded therefore, since this amylose undoubtedly functions as an activator, that a branched structure is not essential for activation.

The difference in structure between amylose and amylopectin may be expressed as a

difference in the number of non-reducing chain ends per unit weight. Whereas in amylose one glucose unit in 200 (average) is a non-reducing end-group, in amylopectin the proportion is about 1 in 20. It would appear therefore that the activating power of a starch polysaccharide is primarily conditioned by the number of chain ends available in unit weight of the activator.

It is convenient, when speaking of the branched structure of amylopectin, to follow Myrbäck and refer to a chain between two branch points as an " inner " chain, an " outer " chain being that between a branch point and a non-reducing end. Limit dextrin-A (which is formed from amylopectin by β-amylolysis), although possessing the same number of terminal non-reducing glucose units per molecule as had the parent amylopectin, has lost the outer chains. The effect of this removal of the outer chains (average length, 10—12 glucose units) on the power to act as primer in the P-enzyme synthesis is illustrated in Table I. Dextrin-A was only one-tenth as active as the amylopectin from which it was derived, an indication that the inner chains (common to amylopectin and its derivative dextrin-A) are not nearly so effective in the activating mechanism as are the outer chains.

A number of bacterial polysaccharides including the dextran synthesised by *Leuconostoc dextranicum* were shown to have no activating power in the synthesis of starch by P-enzyme. Dextran is a polyglucose in which glucose residues are mutually joined mainly by 1 : 6-glucosidic linkages, and its failure, in the usual concentration, to act as a primer in starch synthesis demonstrates that in activating chains the glucose units must be linked as in maltose, *i.e.*, by α-1 : 4-glucosidic bonds. Proehl and Day (*J. Biol. Chem.*, 1946, **163**, 667) and Swanson and Cori (*loc. cit.*), however, find that bacterial dextran in high concentration has a slight activating power. On the other hand, Weibull and Tiselius (*Ark. Kemi, Min. Geol.*, 1945, **19***A*, No. 1) report dextran as being inactive.

The preliminary experiments recorded in Table I also give some indication of the effect of the length of unbranched chains on activator properties. The synthetic amylose I (B.V., 1·33) was prepared in a P-enzyme digest deficient in primer, *i.e.*, under conditions favourable for the production, according to Cori's theory, of chains of maximum length. The synthetic amyloses II and III resulted from syntheses in the presence of excess of primer and, although presumably unbranched, were of much shorter average chain-length than amylose I, as was shown by the respective blue values. Of these, the longest chain amylose (I) had the lowest activating power. Indeed, the activating power appeared to vary inversely as the blue value of the amylose.

Summarising, it is seen that to function as an activator (or primer) in the P-enzyme synthesis of amylose from glucose-1 phosphate, a carbohydrate must be a polyglucose of the starch type in which there are present, either as individuals or as part of a ramified structure, terminated chains of glucose residues joined by α-1 : 4-links. Amylopectin-free amylose is constituted entirely of such chains whereas only the " outer " chains of amylopectin conform to this description.

It was early realised that a factor of equal importance with the number of activating chains available was the actual length of an activating chain. It became apparent that chains containing a minimum number of α-1 : 4-linked glucose units constitute an essential feature of activator molecules. It will be convenient to refer to these terminated unbranched chains (for example, the " outer " chains of amylopectin) as template chains, and the function of P-enzyme is to be pictured as the extension of these template chains by the endwise apposition (by 1 : 4-linkage) of glucose units derived from glucose-1 phosphate. A template chain may be an individual chain (as in amylose) or only part of a longer chain (as in amylopectin). The proposition that an activating chain provides a " template " or " mould " for the phosphorylase brings into prominence the question of the minimum length of such a chain.

Weibull and Tiselius (*loc. cit.*) showed that independent unbranched chains of three, four, five, and six glucose units were capable of functioning as activators towards potato phosphorylase, and the evidence of Swanson and Cori (*loc. cit.*) on the activating power of acid-hydrolysed Schardinger dextrins confirmed that chains composed of only 4—5 glucose units could function as template chains for P-enzyme.

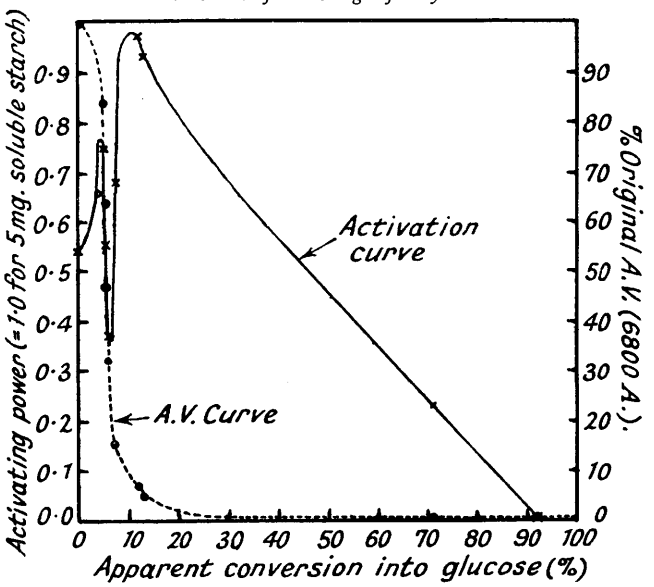
Knowledge of the part played by carbohydrate primers in starch synthesis has been extended by a study of the activating power of the products of hydrolysis of the separated components of starch. These products fell into three groups according as they were obtained from amylose and amylopectin by the agency of (*a*) acid, (*b*) β-amylase, and (*c*) P-enzyme.

Activating Power of the Products of Acid Hydrolysis.—The course of hydrolysis of a starch polysaccharide by acid is two-fold. There occur (i) a rapid fragmentation of the polysaccharide chains into shorter-chain dextrins and (ii) the slower hydrolysis of these dextrins with the liberation of glucose.

(a) *Amylose.* The course of hydrolysis of thymol-amylose (B.V., 1·17) by sulphuric acid is shown by the " A.V.–conversion " curve of Fig. 1. The A.V.–conversion curve is that obtained when the absorption value (6800 A.) of the solution at a given stage in the hydrolysis is plotted (as a percentage of the original A.V.) against the reducing power (expressed as % conversion into glucose) at the same stage (see Bourne, Macey, and Peat, *J.*, 1945, 882). The initial rapid fall of A.V. (6800 A.), during which the blue coloration with iodine almost entirely disappears, represents the fragmentation into dextrins, to which the small increase in reducing power may be mainly ascribed. The second phase, the hydrolysis of dextrin to glucose, becomes predominant at the inflection point and is characterised by a rapid increase of reducing power with very little change in A.V. There is good reason to believe that the average chain-length of the dextrins at the inflection point is about 6. Dextrins of shorter chain-length than 6 are achroic.

FIG. 1.

The effect of acid hydrolysis on the activating power of amylose (B.V., 1·17).
Products from 5 mg. of amylose.



The P-enzyme activating power (relative to soluble starch) of a solution of amylose submitted to hydrolysis with hot N-sulphuric acid was determined at different stages of hydrolysis and plotted, also in Fig. 1, against % apparent conversion into glucose. It is seen that during the initial stages of hydrolysis the activating power of the solution increases rapidly with but little development of reducing power. An overall maximum, which is nearly twice the activating power of the original amylose solution, is attained at a stage when the reducing power of the solution is equivalent to an apparent conversion into glucose of only 10% and when the absorption value has fallen to about one-tenth of its original value. Two maxima are actually featured in Fig. 1. The relative activating power rises from 0·54 to the first maximum at 0·77, and then falls abruptly to 0·35. This fall coincides with the vertical part of the A.V.–conversion curve when the absorption value falls steeply with an almost negligible increase in reducing power. Thereafter the activation curve rises steeply to the second and higher peak at 10% conversion. Finally, the activating power diminishes continuously as the second phase of acid hydrolysis becomes predominant and the template chains are converted into non-activating glucose.

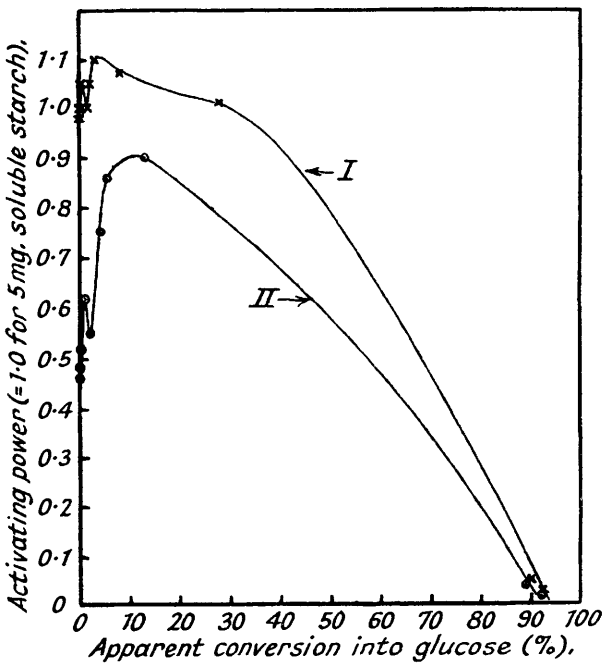
If it be assumed that, in the early stages, but little of the reducing power is due to the liberation of mono- or di-saccharides, then the minimum average length of the amylose fragments can be roughly calculated. Thus the achroic stage is reached at 20% apparent conversion into glucose, so that the average chain-length of the dextrins at this stage is 5 glucose units. This is in agreement with the commonly held view that dextrins of shorter chain-length than 6 or 7 give no colour with iodine. It should be noted that the achroic dextrins at 20% conversion still have a high activating power (0·8), and even at 80% conversion the activating power is still appreciable (0·14).

Swanson and Cori (*loc. cit.*, Fig. 1) observe a similar rise and fall in the activating power of acid-hydrolysed amylose. Only one peak is indicated, however, and this occurs at about 30% apparent conversion into glucose. The activating dextrins at this peak (glucose and maltose were removed by fermentation) were not more than 7 or less than 3 units long.

(b) *Amylopectin.* The effect of acid treatment on the activating power of amylopectin is shown in Fig. 2. This sample of amylopectin had B.V., 0·15, and contained a minimum of unbranched amylose impurity. Curve I (Fig. 2) shows that a small increase in activating power occurs in the earliest stage of hydrolysis, a peak being observed at about 5% apparent conversion into glucose. The curve is distinguished from that of amylose in the same concentration (see Fig. 1) by the much slower rate of fall of activating power as hydrolysis proceeds.

FIG. 2.

The effect of acid hydrolysis on the activating power of amylopectin (B.V., 0·15).



Curve I: Activation tests on products from 5 mg. of amylopectin.
„ II: „ „ „ 0·5 mg. „

It appeared that, in the region of maximum activation represented by the flattened peak of curve I, there existed in the solution a sufficiency of template chains (" outer " chains) to saturate the P-enzyme. After the attainment of maximum activating power, further increase in the number of template chains (by further acid fission) merely provided an excess. The experiment was therefore repeated with a solution of amylopectin only one-tenth as concentrated. Saturation of the P-enzyme was thus avoided, with the result that the activity peak was much sharper (curve II, Fig. 2).

It could be concluded from the sharpness of the amylose activation peak (Fig. 1) that in this case the number of template chains available was insufficient to saturate the P-enzyme at any stage in the hydrolysis, and that the real comparison between amylose and amylopectin is to be made with the amylopectin so diluted that the number of end groups (*i.e.*, template chains) is approximately the same as provided by the amylose. This is achieved when the weight ratio amylose/amylopectin is 10 : 1. The experiments of Swanson and Cori (*loc. cit.*) show a very slow change of activating power with degree of hydrolysis, and we would suggest that this may possibly be due to a saturation of the enzyme at the concentrations used.

There is a striking similarity of form between the activation curves of amylose (5 mg.) and amylopectin (0·5 mg.), a similarity which extends even to the display of two maxima (Fig. 1 and curve II, Fig. 2).

There will be two competing effects of the fragmentation of starch by acid hydrolysis : (i) an increase in the number of template chains, and (ii) a decrease in their average length. Factor (i) will operate immediately, and will increase the activating power, whereas factor (ii) will influence the activating power in the opposite sense by a shortening of the template chains to lengths below the minimum for activation. It is to be expected that factor (ii) will become effective at a later stage than (i). The result will be an initial rapid increase in activating power followed by a slower fall as the extent of hydrolysis increases.

The appearance of two maxima in each case is more difficult to understand. It would suggest that a third factor is involved, namely the existence of an optimum as well as a minimum length for a template chain. The first peak in the amylose curve (Fig. 1) is clearly defined and very sharp because, after the maximum has been attained, the activating power falls abruptly and this fall occurs with practically no increase in reducing power. In other words, the fall from the maximum activating power is coincident with the scission of long chains into shorter chains, the latter being sufficiently long, however, to have very little reducing power. The overall rise in activating power due to increase in the number of template chains is interrupted, on this view, by a fall which is due to the concomitant shortening of the chains to less than the optimum length. This optimum is probably fairly high. Since the reducing power at the first peak is 5% of that of glucose, the average length of the amylose fragments at this stage is at least 20 glucose units.

The first peak in the amylopectin Curve II (Fig. 2) is very small and might easily be overlooked. Were it not that it occurs in both curves I and II it might be ascribed to experimental error. It is in any case not significant in comparison with the first amylose peak. The essential fact is that there is a marked initial increase in the activating powers of acid-hydrolysed amylopectin and amylose, and that this increase is to be ascribed to an increase in the number of template chains in both components.

Activating Power of the Products of β-Amylolysis.—In determining the manner in which activating power might vary with the length of the template chains, β-amylolysis suggested itself as a better approach than acid hydrolysis for the reason that β-amylase does not bring about fragmentation of the substrate into dextrins. If the action of this enzyme results solely in the shortening of the template chains by the separation of maltose units from the non-reducing ends and if, further, it be assumed that all the available chains are equally open to attack, then it follows that β-amylolysis will not change the number of template chains but will diminish the average chain-length.

Certain evidence has been presented (Swanson, *J. Biol. Chem.*, 1948, **172**, 805; Cleveland and Kerr, *Cereal Chem.*, 1948, **25**, 139) which suggests that β-amylolysis may proceed, not by a simultaneous hydrolysis of all the chains available, but by a mechanism which involves the complete conversion of one chain into maltose before a second chain is attacked. The relative merits of these alternative hypotheses will be discussed in a later paper : in our opinion the facts of β-amylolysis are better accommodated by the first.

(a) *Amylopectin.* The variation of activating power with the extent of β-amylolysis of a solution of purified amylopectin (B.V., 0·15) is shown by curve I in Fig. 3. The activating power in general decreases rapidly as hydrolysis by β-amylase proceeds, and becomes very small when approximately half of the amylopectin has been converted into maltose. This agrees with the earlier observation that limit dextrin-A has very little activating power (Table I).

This general decrease in activating power is, however, preceded by a small rise (during the initial stage of β-amylolysis), a maximum being attained at about 5% conversion into maltose. This rise in activating power is made more obvious when the concentration of the original amylopectin solution is suitably diminished to avoid saturation of the P-enzyme by activator molecules (Curve II, Fig. 3).

(b) *Amylose.* The effect of β-amylolysis on the activating power of an amylose solution is also shown in Fig. 3. Curve III relates to a thymol-amylose of B.V. 1·07, which contained some of the branched component, and it is seen that the activating power of the amylose solution falls steadily with increasing hydrolysis until some 35% of the amylose has been converted into maltose. At this point there appears a marked arrest in the diminution of activating power which is maintained until 60% hydrolysis has occurred. Thereafter the activating power actually rises, and a maximum is attained at 80% conversion. Finally the curve falls away and the activating power becomes almost zero (at 92% conversion).

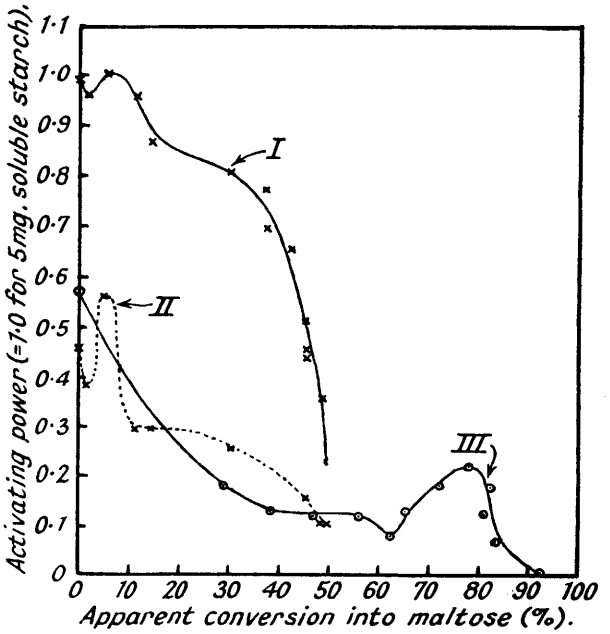
It is clear from this curve that a shortening of the amylose chain by the step-by-step removal of maltose from the reducing ends results in a diminution in P-enzyme activating power until a stage is reached represented by a conversion of 40—50% of the amylose into maltose. The

flattening of the curves could be ascribed to the existence of an optimum length of template chain which begins to be effective in the region of 40—50% conversion whereby an increase in activating power (as the average chain length approached the optimum) is superimposed upon the normal fall which would occur in the absence of an optimum.

On the basis of blue value it is calculated that an amylose of B.V. 1·07 contains not more than 30% amylopectin, and Curve I shows that this amylopectin would be almost inactivated by hydrolysis before the region of 40—50% conversion were reached. It can be accepted therefore that the activity peak at 80% conversion is mainly due to the unbranched chains of the amylose hydrolytic products. It can be calculated (when allowance is made for the presence of 20—30% amylopectin) that, if the chain-length of the original amylose is 200, the average chain length of the residual dextrins when 80% of the amylose (B.V., 1·07) has been converted into maltose will be 15—25 glucose units. This value agrees with that deduced from the acid hydrolysis curve (Fig. 1) for the optimum length of the template chain.

FIG. 3.

The effect of β-amylolysis on the activating power of amylose and amylopectin.



Curve I: Products from 5 mg. of amylopectin (B.V., 0·15).
„ II: „ „ 0·5 mg. „ „
„ III: „ „ 5 mg. of amylose (B.V., 1·07).

Phosphorolytic Degradation of Amylose and Amylopectin.—The phosphorolytic function of P-enzyme has not been so closely studied as has its synthetic function. The kinetics of the whole system, both synthetic and degradative, were examined by Hanes, and much data concerning phosphate equilibrium ratios were provided. The substrate used by Hanes was, however, whole starch, and it became desirable to investigate the phosphorolysis of the separated components.

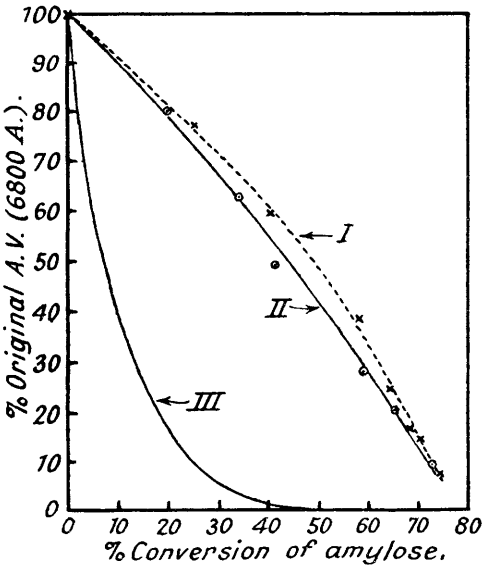
(a) *Amylose.* Repeated experiments established that P-enzyme preparations which were highly active in synthesis had no degradative action on amylose if phosphate ions were absent. In the presence of inorganic phosphate, however, amylose was readily converted by P-enzyme into glucose-1 phosphate. This phosphorolysis has many features in common with β-amylolysis of amylose. The progress of phosphorolysis was followed by the simultaneous estimation of % of original A.V. and % conversion into ester phosphate, and the A.V.–conversion curve compared with those for β-amylase and α-amylase (Fig. 4). The curves for P-enzyme (I) and β-amylase (II) are identical in form and almost coincidental. The amylose used in the two experiments had B.V. 1·07 and, as already stated, contained 20—30% amylopectin. The fact that, as shown by Fig. 4, the conversion of this amylose into glucose phosphate or maltose is not 100% may be explained as due to the presence of amylopectin in the above proportion.

Evidence is thus provided that degradation of amylose by P-enzyme proceeds by a mechanism similar to that of β-amylolysis, in that both methods of attack engage non-reducing chain-ends and split off single units (glucose phosphate or maltose) from these ends. α-Amylolysis follows a widely different course (see Curve III, Fig. 4).

(b) *Amylopectin.* It has been shown that P-enzyme catalyses the scission, as well as the formation, of α-1 : 4-linkages. Under certain conditions this enzyme effects the synthesis of unbranched chains but not of branched structures of the amylopectin type. P-Enzyme being incapable of establishing cross linkages, it is reasonable to suppose that neither can it catalyse the scission of such links. If this is so it can be inferred that complete phosphorolysis of natural amylopectin is not possible. Preliminary quantitative tests showed that amylopectin was partly degraded by P-enzyme, but only in the presence of inorganic phosphate. Consequently, in the later experiments, the digests all contained an adequate excess of phosphate to ensure the complete conversion, if it were possible, of the polysaccharide into glucose-1 phosphate.

FIG. 4.

The phosphorolysis and amylolysis of amylose (B.V., 1·07).



By (a) *P-Enzyme (to ester-phosphate).—Curve I.*
(b) *β-Amylase (to maltose).—Curve II.**
(c) *α-Amylase (to maltose).—Curve III.*†

* *Provided by Dr. W. J. Whelan.*
† *From Bourne, Macey, and Peat (J., 1945, 882).*

These experiments established that the cross-links in amylopectin obstruct P-enzyme in its degradative function in the same way as they act as a barrier to the further action of β-amylase. The arrest point is not, however, the same for the two enzyme systems. Whereas β-amylolysis ceased at 53% conversion (into maltose), P-enzyme carried the phosphorolysis to a limit of 39% conversion (into glucose phosphate). The limit dextrins (named in this paper dextrin-A and dextrin-P respectively) both gave red stains with iodine and showed the same blue value (0·19).

It was further shown that the higher degree of hydrolysis effected by the β-amylase was not due to the contamination of the latter enzyme with α-amylase inasmuch as the dextrin-A was not susceptible to attack by β-amylase whereas the dextrin-P was. β-Amylolysis of dextrin-P brought about a further conversion of 18% (based on weight of original amylopectin) into maltose.

It is clear that phosphorolytic degradation does not shorten the outer chains of amylopectin to the same extent as does β-amylolysis. Indeed, at 40% conversion, when P-enzyme action ceases, the shortened outer chains are still long enough to function as templates for the synthetic action of P-enzyme. This is shown by the fact that dextrin-P is 6—7 times more active as a primer than is dextrin-A (see Table I).

The approximate length of the template chains of dextrin-P can be calculated from the conversion figures. Accepting 20 as the average number of glucose residues in the repeating chain unit of amylopectin, it follows from the fact that dextrin-P is converted into dextrin-A by β-amylase with the concomitant liberation of 18% by weight of the original amylopectin as maltose that the repeating chains of dextrin-P are, on the average, longer than those of dextrin-A by 3—4 glucose units. What may be called the outer chains of dextrin-A are composed of at least one, and possibly two, glucose members (Haworth, Kitchen, and Peat, *J.*, 1943, 619), so that the outer chains of dextrin-P contain at least 5—6 glucose units. Further evidence is thus afforded that a chain of as few as 6 units will function as a template chain.

Experimental.

Sources of the Enzymes.—The salivary α-amylase employed in this work was prepared by the procedure of Bourne, Haworth, Macey, and Peat (*J.*, 1948, 924), and was free from maltase. The β-amylase, isolated from soya beans, showed neither α-amylase nor maltase activity (see Bourne, Macey, and Peat, *loc. cit.*). The preparation of amylase-free potato phosphorylase (P-enzyme) has been described earlier (Bourne and Peat, *J.*, 1945, 877).

Sources of the Carbohydrate Activators.—(a) *Maltose.* This was a highly purified specimen obtained by fractional crystallisation from commercial maltose.

(b) *Acid-solubilised starch.* A commercial sample of acid-solubilised starch was used as a standard primer for the synthesis of starch-type polysaccharides from dipotassium α-D-glucopyranose-1 phosphate.

(c) *Amylose and amylopectin.* These were derived from native potato starch by selective precipitation of the amylose as a complex with thymol or *cyclo*hexanol (Haworth, Peat, and Sagrott, *Nature*, 1946, **157**, 19; Bourne, Donnison, Haworth, and Peat, *J.*, 1948, 1687) and by adsorption of the amylopectin on an aluminium hydroxide gel (Bourne, Donnison, Peat, and Whelan, *J.*, 1949, 1).

(d) *Dextrin-A* (*α-amylodextrin*). Two samples of dextrin-A were prepared, one from native potato starch and one from thymol-amylopectin, by β-amylolysis under the conditions prescribed by Bourne, Haworth, Macey, and Peat (*loc. cit.*).

(e) *Synthetic "amyloses"* (I, II, and III). These "amyloses" were unbranched polysaccharides synthesised by amylase-free P-enzyme from dipotassium glucose-1 phosphate (220 mg.) in the presence of varying proportions (0·5, 60, and 120 mg.) of a red-staining activator prepared from potato amylose by acidic hydrolysis.

(f) *Dextrin-P.* Dextrin-P was the limit dextrin produced by amylase-free P-enzyme from amylopectin in the presence of excess of phosphate buffer.

(g) *Dextran.* The sample of bacterial dextran was kindly supplied by Professor M. Stacey.

Measurement of the Iodine Stains of Polysaccharides.—The two expressions, absorption value (A.V.) and blue value (B.V.), used to describe the intensities of the iodine stains of polysaccharides were defined by Bourne, Haworth, Macey, and Peat (*loc. cit.*). Whereas the B.V. is determined under standard conditions of concentration and wave-length of light and is therefore characteristic of the polysaccharide in question, the A.V. is simply a convenient term for a measurement of relative intensity made under any conditions.

Determination of Reducing Sugar.—Shaffer and Hartmann's copper reagent (*J. Biol. Chem.*, 1921, **45**, 377) was used for the determination of both glucose and maltose. For the determination of very small amounts ($< 0·2$ mg. of glucose and $< 0·4$ mg. of maltose) a known weight of the appropriate sugar was introduced, and the amount originally present was calculated by difference.

Determination of Inorganic Phosphorus.—A colorimetric method (Allen, *Biochem. J.*, 1940, **34**, 858) was used for the determination of inorganic phosphorus. Calibration of the reagents against potassium dihydrogen phosphate gave a linear graph when the absorption value measured on a Spekker absorptiometer was plotted against the weight of phosphorus present. This graph showed the method to be adequate for the determination of 0·02—0·20 mg. of inorganic phosphorus.

Standard Procedure for the Measurement of the Activating Power of a Carbohydrate.—A modification of the method employed by Green and Stumpf (*loc. cit.*) for the determination of phosphorylase activity was used for the measurement of the activating power of a carbohydrate. A standard digest containing citrate buffer (pH 6·0; 1·0 c.c.), dipotassium glucose-1 phosphate (0·1M; 1·0 c.c.), the activator solution (1·0 c.c., containing 5 mg. of carbohydrate), and P-enzyme solution (0·5 c.c.), the last being added to the remainder of the digest only when the temperature had reached 35·5°, was incubated at 35·5° for 12 minutes. The synthesis was arrested with 6% trichloroacetic acid solution (5 c.c.), the protein precipitate removed on the centrifuge, and an aliquot portion of the supernatant liquid analysed colorimetrically for inorganic phosphorus. A simultaneous control digest, containing the same solutions except that the activator solution was replaced by an equal volume of water, served to measure the extent to which the synthesis had been primed by natural activators present in the glucose-1 phosphate, which had been prepared enzymically from potato starch. The synthesis achieved in this control was always small in comparison with that in the main digest. The increase in inorganic phosphorus attributable to the carbohydrate under test was found by difference.

The activating power of the carbohydrate primer relative to that of commercial soluble starch, determined under the same conditions, is given as the ratio of the weight of phosphorus liberated in the presence of 5 mg. of carbohydrate to the weight liberated in the presence of 5 mg. of soluble starch.

Effect of Acid Hydrolysis on the Activating Power of Amylose.—Thymol-amylose (500 mg.; B.V. 1·17) was dissolved in hot water (45 c.c.) containing a little sodium hydroxide. The solution was cooled, neutralised with sulphuric acid, diluted to 50 c.c., and mixed with 2N-sulphuric acid (50 c.c.). It was placed under a reflux condenser in a water-bath at 75°, and the temperature of the bath was then slowly raised to 100°. In this way the initial stages of the hydrolysis could be studied more closely than would have been possible had the reaction been started at 100°. At selected stages of the reaction an aliquot

volume (5 c.c.) was removed and adjusted to *ca.* pH 6·0 with sodium hydroxide, the final volume being 10 c.c. The following analyses were made on each neutral sample :

(*a*) A portion (2 c.c. ≡ 5 mg. of amylose) was added to water (200 c.c.), acidified with 6N-hydrochloric acid (3 drops), stained with a solution (5 c.c.) of 0·2% iodine in 2% potassium iodide, and diluted to 500 c.c. The A.V. (6800 A.) of this coloured solution was determined.

(*b*) The reducing power of a second portion (2 c.c. ≡ 5 mg. of amylose) was measured by the Shaffer–Hartmann method.

(*c*) A third portion (2 c.c. ≡ 5 mg. of amylose) was tested as an activator of the synthetic function of P-enzyme. Since only 1 c.c. of activator solution was normally used in the standard method it was necessary to dissolve the glucose-1 phosphate directly in the citrate buffer to preserve the usual concentrations.

The results of this experiment are shown graphically in Fig. 1.

Effect of Acid Hydrolysis on the Activating Power of Amylopectin.—An amylopectin sample (B.V., 0·15), prepared from potato starch by the *cyclo*hexanol–methyl alcohol method, was hydrolysed with acid under the conditions which have already been described in the case of amylose. In a duplicate experiment a ten-fold dilution (≡ 0·5 mg. of amylopectin) of the neutralised hydrolysate was used in the activation tests. The results are recorded in Fig. 2.

Effect of β-Amylolysis on the Activating Power of Amylose.—A solution of thymol-amylose (500 mg.; B.V., 1·07) in water containing a little sodium hydroxide was neutralised with sulphuric acid, diluted to 50 c.c., and mixed with citrate buffer (pH 6·0; 25 c.c.) before being incubated at 36° with a 0·3% solution (25 c.c.) of soya-bean β-amylase. At intervals, aliquot volumes (5 c.c.) of the digest were withdrawn, inactivated by immersion in a boiling water-bath for 4 minutes, and used for the following measurements :

(*a*) A portion (1·0 c.c. ≡ 5 mg. of amylose) was subjected to the Shaffer–Hartmann determination, the reducing power being expressed in terms of maltose.

(*b*) A portion (1·0 c.c. ≡ 5 mg. of amylose) was tested as a primer for the synthetic action of P-enzyme by the standard method. The results are shown in Fig. 3.

Effect of β-Amylolysis on the Activating Power of Amylopectin.—The previous experiment was repeated on amylopectin (B.V., 0·15) prepared from potato starch by the *cyclo*hexanol–methyl alcohol method. In a duplicate experiment a ten-fold dilution (≡ 0·5 mg. of amylopectin) of each inactivated test sample was used for the activating power measurement. The results are recorded in Fig. 3.

Phosphorolysis of Amylose.—A solution of thymol-amylose (96 mg.; B.V., 1·07) in water containing a little sodium hydroxide was neutralised with sulphuric acid, diluted to 40 c.c., and mixed with a 0·2M-phosphate buffer (40 c.c.; pH 6·4). The solution was incubated at 35·5° with an amylase-free P-enzyme solution (16 c.c.), aliquot volumes being removed at intervals for the following measurements :

(*a*) A digest sample (1·0 c.c. ≡ 1·0 mg. of amylose) was diluted with water (50 c.c.), slightly acidified with hydrochloric acid, stained with a solution (1·0 c.c.) of 0·2% iodine in 2% potassium iodide, and diluted to 100 c.c. The A.V. (6800 A.) was then measured.

(*b*) The reducing power of a sample (5·0 c.c.) was determined by the Shaffer–Hartmann method. No reducing sugars were formed throughout the period of digestion.

(*c*) A portion (5 c.c.) of the digest was boiled for 30 seconds, cooled, and mixed with a solution (5 c.c.) of magnesium acetate (0·09 g.). Ammonia solution was added to precipitate the bulk of the inorganic phosphate as magnesium ammonium phosphate, which was removed by filtration and washed with water. The filtrate and washings were diluted with water to 100 c.c., and determinations of inorganic and total phosphorus were made on this solution. The amount of ester phosphorus was calculated by difference. Control experiments on known mixtures of glucose-1 phosphate and the phosphate buffer in proportions similar to those present at different stages in the above digest showed that the ester phosphorus determination was correct to within 2%. The results are shown graphically in Fig. 4.

Phosphorolysis of Amylopectin.—A digest containing a dispersion of amylopectin (180 mg.; B.V., 0·15) in water (90 c.c.), phosphate buffer (0·2M; pH 6·4; 75 c.c.), and amylase-free P-enzyme solution (15 c.c.) was incubated at 35·5°. At intervals the reducing power and ester phosphorus were determined by the methods used in the previous digest. The results are given in Table II.

TABLE II.

Phosphorolysis of Amylopectin (B.V., 0·15).

Time of incubation (min.).	Apparent conversion (%) into maltose (from R_{Cu}).	Conversion (%) of amylopectin into ester phosphate.
0·5	Nil	0·0
30	,,	29·8
126	,,	39·7
260	,,	39·2
325	,,	38·7

After 325 minutes the digest was inactivated by boiling, and cooled. An aliquot portion (10 c.c.) was incubated at 35·5° with a 0·25% solution (10 c.c.) of soya-bean β-amylase, and the reducing power was determined at intervals by the Shaffer–Hartmann method. The conversion into maltose, calculated on the original amylopectin, was 12·5% (1 hour) and 18·1% (22 hours).

The authors wish to express their thanks to Professor Sir Norman Haworth, F.R.S., for his interest, and to the Colonial Products Research Council for financial aid to one of them (D. A. S.). They are grateful also for the assistance of Mr. S. A. Barker, a member of this research group.

THE UNIVERSITY, EDGBASTON, BIRMINGHAM, 15. [*Received, August 19th,* 1948.