705. *The Enzymic Synthesis and Degradation of Starch. Part X. The Phosphorylase and Q-Enzyme of Broad Bean. The Q-Enzyme of Wrinkled Pea.*

By P. N. Hobson, W. J. Whelan, and Stanley Peat.

Q-Enzyme, which converts amylose into amylopectin and which, with phosphorylase and glucose-1 phosphate, is responsible for the synthesis of amylopectin, has been isolated (as a stable freeze-dried powder) from the broad bean and the wrinkled pea. These preparations exert the same function as the Q-enzyme of potato. The phosphorylase of bean has also been isolated and shown to be closely similar to that of the potato. It is concluded that the mechanism of starch synthesis in these three plants is the same.

Methods of removing α-amylase impurity from phosphorylase and Q-enzyme preparations have been investigated and a reliable test for α-amylase in the presence of other enzymes is described. The phosphorylase has been almost entirely freed from α-amylase, but a complete separation of this impurity from Q-enzyme has not been achieved.

In the course of these investigations the presence of a " debranching " enzyme has been established. This will be the subject of a later communication.

The synthesis of starch in the potato is effected by two enzymes, namely, phosphorylase, which synthesises amylose from glucose-1 phosphate, and Q-enzyme, which, with phosphorylase, effects the synthesis from glucose-1 phosphate of the ramified polysaccharide, amylopectin, and which also, when acting alone, converts amylose into amylopectin. Potato phosphorylase was described by Hanes in 1940 (*Proc. Roy. Soc.*, **129**, *B*, 174) and Q-enzyme of potato in 1944 by Haworth, Peat, and Bourne (*Nature*, **154**, 236). The properties of potato Q-enzyme have been the subject of several papers in this series by Peat and his co-workers (*J.*, 1945, 877, 882; 1949, 1705, 1712; 1950, 84, 93).

The phosphorylases of the pea (Hanes, *Proc. Roy. Soc.*, 1940, **128**, *B*, 421), waxy maize (Bliss and Naylor, *Cereal Chem.*, 1946, **23**, 177), lima bean (Green and Stumpf, *J. Biol. Chem.*, 1942, **142**, 355), barley (Porter, *Biochem. J.*, 1949, **45**, xxxvii), and jack bean (Sumner, Chou, and Bever, *Arch. Biochem.*, 1950, **26**, 1) have already been described, but Q-enzyme has been obtained hitherto only from the potato. We have now made a detailed examination of the starch-synthesising enzymes of the broad bean (Sutton's " Prolific Longpod ") and have compared them with the purest available samples of the corresponding potato enzymes. It has been found possible to apply to the bean the methods of lead-complex precipitation and ammonium sulphate fractionation used in the isolation of potato enzymes by Barker, Bourne, Wilkinson, and Peat (*J.*, 1950, 84). Bean phosphorylase and Q-enzyme have been isolated as stable freeze-dried powders at the P1 and Q3 stages in the fractionation scheme set out by the above authors. These preparations have been used for comparative purposes and also as the starting materials for attempts at further purification.

Properties of the Bean Q-Enzyme.—The action of bean Q-enzyme (Q3 fraction) on amylose presents the same features as those which characterise the action of potato Q-enzyme. The reaction shows two distinct phases. There is an initial rapid fall in the blue value of amylose (on average, from 1·40 to *ca.* 0·2), during which the colour of the iodine stain changes from blue

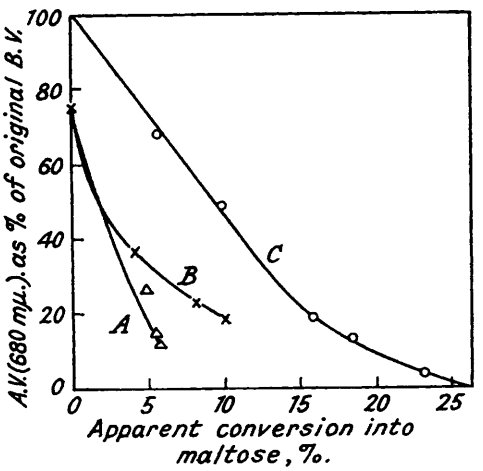
Action of bean Q-enzyme on amylose.

Period of incubation with Q-enzyme, mins.	0	180	635	1275
B.V. of polysaccharide	1·43	0·720	0·279	0·143
Limiting conversion into maltose, % { 5 hrs.	90·5	72·0	63·2	53·8
Limiting conversion into maltose, % { 10 hrs.	90·5	72·3	63·4	54·6

through purple to red. This is followed by a further fall in blue value, but this secondary change takes place at a much diminished rate. At the same time a small copper-reducing power develops. The relationship between intensity of iodine stain and reducing power is depicted in Fig. 1, which also shows the corresponding curves for the Q-enzyme of wrinkled pea (see below) and salivary α-amylase. It is seen that the Q-enzymes from bean and pea resemble each other closely but that each is distinct from α-amylase. It has not yet been decided whether the liberation of reducing groups by a Q-fraction is due to an intrinsic action of the enzyme or to the presence in the preparation of a trace of α-amylase (see Part VII; Barker, Bourne, Wilkinson,

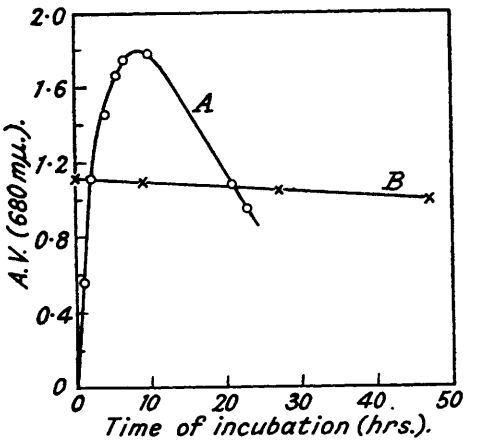
FIG. 1.

The hydrolysis of amylose by Q-enzymes of bean and pea.



A.—Q-Enzyme of wrinkled pea.
B.—Q-Enzyme of broad beans.
C.—Salivary α-amylase.

FIG. 2.



A.—Action of bean phosphorylase on glucose-1 phosphate.
B.—Action of bean phosphorylase on natural amylose (phosphate absent).

and Peat, *J.*, 1950, 93). Experiments described below do in fact show that the bean preparation contained a small amount of α-amylase. It should be noted further that even crystallised potato Q-enzyme liberates some reducing groups from amylose (Gilbert and Patrick, *Nature*, 1950, **165**, 573). The bean preparation was free from maltase. The synthesis of branch linkages by bean Q-enzyme is shown by the results of an experiment in which the Q-enzyme after acting on a pure amylose for varying periods was deactivated (by being boiled), soya-bean β-amylase added, and the limiting conversion of the polysaccharide into maltose determined after 5 hours and after 10 hours (see table above). As the blue value of the amylose decreased, the extent of β-amylolysis of the resulting polysaccharide became smaller until, after incubation for 21 hours with Q-enzyme, a polysaccharide having B.V. 0·143 and a limiting conversion into maltose of 56% remained. The corresponding values for a natural amylopectin are 0·153 and 50%. The action of the bean Q-enzyme on amylose is therefore identical with that of the potato enzyme as reported by Barker, Bourne, and Peat (*J.*, 1949, 1712).

In the following table, a comparison is made, with respect to pH and temperature optima, between Q-enzyme, phosphorylase, and α-amylase of the bean and the corresponding enzymes from potato. A close similarity between these enzymes is evident.

Purification and Properties of Bean Phosphorylase.—This enzyme has been obtained in a highly purified form by the modification of the fractionation procedure of Barker, Bourne,

Temperature and pH optima. Bean and potato enzymes.

	Optimum pH.	Optimum temp.
Bean Q-enzyme	7·25—7·5	20°
Potato Q-enzyme	7·0 *	21±1 *
Bean phosphorylase	5·7—5·8 (for synthesis)	39—41
Potato phosphorylase	5·9—6·3 † (for synthesis)	—
Bean α-amylase	6·1—6·3	—
Potato α-amylase	6·0—6·5 ‡	—

* Barker, Bourne, and Peat, *J.*, 1949, 1712.
† Hanes, *Proc. Roy. Soc.*, 1940, **129**, *B*, 174.
‡ James and Cattle, *Biochem. J.*, 1933, **27**, 1805.

Wilkinson, and Peat (*J.*, 1950, 84) shown in Table I. In the absence of phosphate ions, the action of the purified bean phosphorylase (P5 fraction) on amylose was so slight (21 mg. of the freeze-dried powder had a smaller effect on the blue value of amylose than had 1 c.c. of saliva which had been diluted 30,000 times) that it was evident that Q-enzyme and α-amylase were present only as traces. The powder had a higher phosphorylase activity (112 units per g.) than had a typical preparation from potato (92 units per g.), and the phosphorylase–α-amylase activity ratios for these potato and bean preparations were 53 : 1 and 123 : 1 respectively. The bean phosphorylase was shown, by applying the usual tests, to be free from maltase and invertase. The absence of phosphoglucomutase was indicated by the fact that in the course of synthesis of polysaccharide by the phosphorylase preparation acting on glucose-1 phosphate, the mineral phosphate liberated rapidly attained a constant value which remained unchanged for 56 hours. On the other hand, the absorption value (for definition of A.V., see Bourne, Howarth, Macey, and Peat, *J.*, 1948, 929) of the digest during synthesis rose to a maximum but thereafter diminished rapidly (see Fig. 2). Clearly, degradation of amylose was also occurring independently of the true phosphorylase action. The same observation was made with regard to phosphorylase preparations from potato.

TABLE I.

Isolation of phosphorylase from broad bean extract.

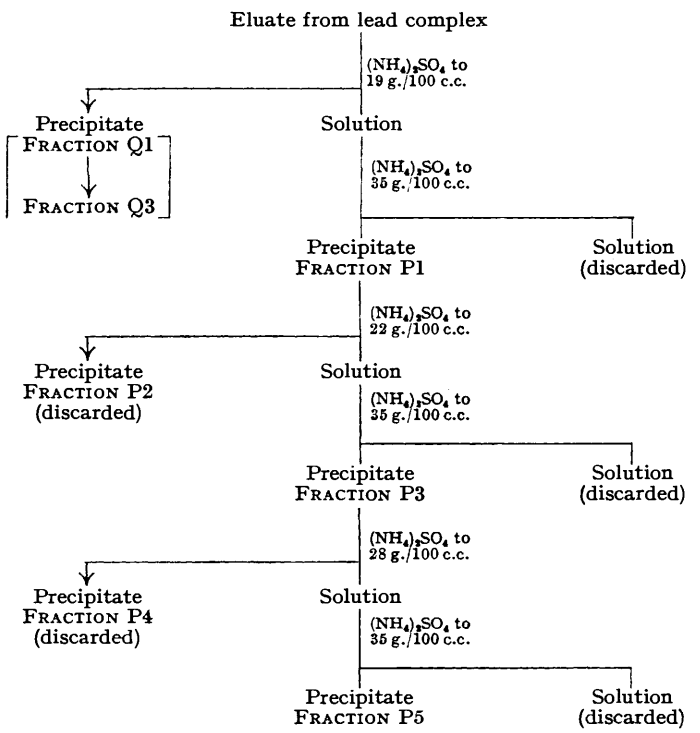


TABLE II.

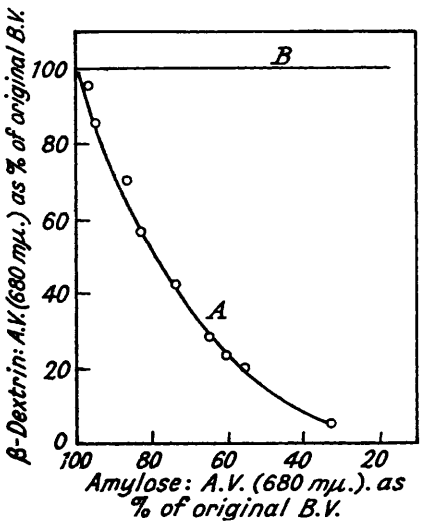
Action of salivary amylase on amylose and amylopectin.

	Amylose.		Amylopectin.	
Time (hrs.).	A.V.	Fall in A.V., %.	A.V.	Fall in A.V., %.
0	1·45	0	0·158	0
6	1·37	4·5	0·087	45·0

The hydrolysis of this synthetic amylose proceeded much more rapidly than that of natural amylose when each was acted upon by equivalent amounts of the phosphorylase preparation (Fig. 2). This difference of rate is to be expected since the rate of fall of the absorption value of a polysaccharide when treated with an α-amylase is related inversely to its chain length. Calculation from the amount of " primer " (achroic dextrins from acid hydrolysis of amylose) used in the phosphorylase digest showed that the average chain length of the synthetic polysaccharide was much less than the chain length of natural amylose, which is at least 200 glucose units. This relationship between rate of α-amylolysis and basal chain length is further stressed by the values given in Table II, which refer to the action of the same amount of freeze-dried salivary amylase on equal weights of amylose and amylopectin.

FIG. 3.

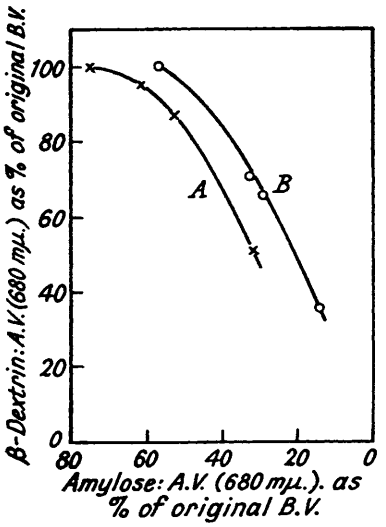
Amylose–β-dextrin curves.



A.—Salivary α-amylase.
B.—Phosphorylase, Q-enzyme, or β-amylase.

FIG. 4.

Fractionation of Q-enzyme.



A.—Q9 fraction.
B.—Q3 fraction.

A New Method of Characterising α-Amylase.—The most persistent enzymic impurity in phosphorylase and Q-enzyme preparations is α-amylase, and we have attempted by various means to remove this impurity. It was, however, necessary first to devise a rapid and unequivocal method of distinguishing α-amylase from the other enzymes. We adopted therefore the procedure of incubating equal amounts of the enzymic solution with (i) amylose and with (ii) β-dextrin, *i.e.*, the limit dextrin from the action of β-amylase on amylopectin (named dextrin-A in earlier communications), and determining the changes taking place in the intensities of the iodine stains. By plotting the % change in absorption value of amylose against that of β-dextrin, the time factor is eliminated and a curve is obtained which is characteristic of the enzyme and independent of its concentration. Because Q-enzyme, phosphorylase (acting in the presence of phosphate), and β-amylase attack amylose but not β-dextrin, a single straight line serves to represent the action of all three enzymes; α-amylase degrades both substrates, and the curve depicting its action is given in Fig. 3. This test was employed in following the course of further attempts to eliminate α-amylase from preparations of Q-enzyme and phosphorylase.

Reprecipitation with Ammonium Sulphate.—A Q3 fraction was dissolved in water and precipitated with ammonium sulphate (19 g./100 c.c.), giving a Q4 fraction. The treatment was

repeated until the Q9 stage was reached. The results of tests for α-amylolytic activity on fractions Q3, Q5, Q7, and Q9 (Fig. 4) showed that the reprecipitation effected no improvement in the Q-enzyme : α-amylase ratio.

Fractional precipitation of bean phosphorylase with ammonium sulphate, by the method illustrated in Table I, did, however, remove a considerable proportion of α-amylase, as may be judged from the fact that amounts of P3 and P5 fractions, equal with respect to phosphorylase activity, showed α-amylolytic activities corresponding to diminutions of 78·2% and 8·9% respectively in the blue value of amylose over a period of 40 hours.

Irradiation with Ultra-violet Light.—The rates of deactivation of purified bean Q-enzyme, bean phosphorylase, and salivary α-amylase when irradiated by ultra-violet light (filtered, 365 mμ., and unfiltered) were compared. The results showed that α-amylase was deactivated much more rapidly than either the Q-enzyme or the phosphorylase. Measurement of the changes in Q-enzyme, phosphorylase, and α-amylase activities produced by the irradiation of aqueous extracts of bean appeared to confirm these results. When, however, the Q-enzyme and phosphorylase were isolated after the bean amylase had presumably been destroyed by irradiation, a small amount of α-amylase still persisted in both products. Thus, like the lead-complex precipitation method, irradiation eliminates most, but still not all, of the amylolytic activity and therefore offers no advantage over the original method of purification.

Treatment with Ozone.—The passage of ozone through an aqueous bean extract caused a selective deactivation of α-amylase, suggesting that ozone generated by ultra-violet light was the deactivating agent in the experiments recorded above. By this method also it was found impossible to remove last traces of α-amylase.

Fractionation on Cellulose and Other Columns.—Solutions of bean phosphorylase (fraction P3) were perfused through columns of cotton wool and powdered cellulose impregnated with dilute amylose solution. The amylose–β-dextrin curves for the effluents indicated that no separation of α-amylase had occurred. Similar negative results were obtained on using a column of whole potato starch. A column of lead phosphate completely absorbed, or deactivated, all the enzymes.

Adsorption on Starch from Dilute Alcoholic Solution.—Holmberg (*Biochem. Z.*, 1933, **258**, 134) devised a method of removing α-amylase from malt amylase by its absorption on starch grains from a 50% alcohol solution. We applied this method to preparations of bean Q-enzyme and phosphorylase in 20% alcohol solution at 0°. Not only was α-amylase removed from the solution but so also were Q-enzyme and phosphorylase. There remained in the supernatant liquid, in the Q-enzyme experiment, an enzyme (R-enzyme) which has the property of hydrolysing the branch linkages in amylopectin and β-dextrin. The same enzyme was subsequently isolated by the same method from the potato. A preliminary account of this enzyme has already been published (Hobson, Whelan, and Peat, *Biochem. J.*, 1950, **47**, xxix).

The Q-Enzyme of Wrinkled Peas.—A Q-enzyme has also been isolated from wrinkled peas (Laxton's " Progress "), and its action on amylose in respect of changes in iodine stain and liberation of reducing groups is shown in Fig. 1. The similarity of the wrinkled pea enzyme to that of the broad bean is evident. It is of interest that Q-enzyme should be found in this plant in view of the very high (98%) amylose content of wrinkled pea starch (see Peat, Bourne, and Nicholls, *Nature*, 1948, **161**, 206).

Experimental.

Analytical Methods.—(*a*) For the methods used in measurement of the iodine stains of polysaccharides, inorganic- and ester-phosphorus, and phosphorylase activity see Part IV (*J.*, 1949, 1705). Blue values (B.V. and A.V.) refer to γ 680 mμ. throughout.

(*b*) *Determination of reducing sugar.* Somogyi's micro-copper reagent (*J. Biol. Chem.*, 1945, **160**, 61) was used. It was shown to be unaffected by the concentrations of citrate or acetate buffers used in the digests. Before being added to the reagent the digest portions were deproteinised as follows. A predetermined amount of 1N-sodium hydroxide was added to make the solution faintly alkaline, followed by zinc sulphate (5%; 0·2 c.c.) and 0·3N-barium hydroxide in equivalent amount (see Somogyi, *ibid.*, p. 69). The protein complex was removed on the centrifuge, and an aliquot of the supernatant liquid added to the copper reagent.

(*c*) *Determination of β-amylase activity.* An amount of the β-amylase, predetermined to liberate 10—20 mg. of maltose in the following digest, was dissolved in water and a portion (2 c.c.) transferred to a mixture of " AnalaR " soluble starch solution (0·6%; 25 c.c.) and 0·2M-acetate buffer (pH 4·8; 3 c.c.), preheated to 35·5°. The digest was incubated at this temperature for exactly 30 mins., and a portion (5 c.c.) was then removed and added immediately to the copper reagent for determination of maltose. The activity of the enzyme is defined as the number of mg. of maltose liberated by 1 mg. of enzyme preparation under the above conditions. Soya-bean β-amylase, prepared as in Part II (*J.*, 1945, 882), was used throughout.

Preparation of Starch Fractions.—Amylose was separated from potato starch either by precipitation with thymol (Bourne, Donnison, Haworth, and Peat, *J.*, 1948, 1687) or by the aluminium hydroxide method (Bourne, Donnison, Peat, and Whelan, *J.*, 1949, 1). The amylopectin was initially separated from potato starch by the thymol method and further purified by repeatedly passing a neutral solution (0·1%; 250 c.c.) through a column of defatted cotton wool (100 × 1·5 cm.) until no further fall in A.V. occurred. The amylopectin was isolated by precipitation with ethyl alcohol (2 vols.) and dried by trituration with alcohol and ether.

Preparation of β-Dextrin (*syn. Dextrin-A*).—Amylopectin (4·5 g.; B.V. 0·19) was wetted with alcohol, dissolved in N-sodium hydroxide (50 c.c.) by warming on a water-bath, cooled, and neutralised to phenolphthalein with dilute sulphuric acid. Acetate buffer (0·2M.; pH, 4·8; 30 c.c.) and soya β-amylase (in weight equivalent to 6250 units of activity; 40 c.c.) were added, followed by water to 250 c.c. The digest was incubated at 35·5° for 24 hours and then boiled for 10 minutes. The solution was dialysed in " Cellophane " tubing against running tap water for 72 hours and then concentrated to *ca.* 150 c.c. by distillation under diminished pressure. The above digest was reconstituted by the addition of enzyme, buffer, and water to the concentrated solution, and the entire procedure repeated. The concentrated solution was finally freeze-dried in a high vacuum. The β-dextrin so prepared was a white fibrous solid; average yield, 1·5 g.

Ammonium sulphate solution was prepared by dissolving the " AnalaR " salt in water to a concentration of 50 g./100 c.c., filtering, and adjusting the pH to 7·0 with ammonia solution (*d* 0·88). It was cooled to 0° before use.

Standard Method for the Isolation of Bean Phosphorylase and Q-Enzyme.—Broad beans (Sutton's " Prolific Longpod ") were ground to a fine flour and defatted with ether by continuous washing in a Buchner funnel until the washings were colourless; ether was then removed by suction. The flour (300 g.) was extracted with water (1200 c.c.) by shaking at room temperature for 30 minutes, *sec.*-octyl alcohol (5 drops) being added to prevent foaming. The flour was removed on a centrifuge, and from the supernatant liquid a lead–protein complex was precipitated and then eluted by using the method given in Part IV (*loc. cit.*). The eluate was cooled to 0°, and ammonium sulphate solution added with stirring to bring the salt concentration to 19 g./100 c.c. The solution was stored at 0° until the protein precipitate had begun to flocculate (about 6 hrs.). This precipitate (fraction Q1) was removed on a centrifuge and dissolved in water (100 c.c.). The Q-fraction was precipitated (ammonium sulphate added to 19 g./100 c.c.), stored for 30 minutes, the precipitate removed, dissolved in 0·1M-citrate buffer (pH 7·0; 50 c.c.), and freeze-dried in a high vacuum. To the supernatant liquid from the Q1 precipitation was added ammonium sulphate to a concentration of 35 g./100 c.c., and after 2 hours the precipitate (P1-fraction) was removed on a centrifuge. This fraction was either dissolved in buffer (as for Q-enzyme) and freeze-dried or used for the preparation of P5-fraction according to the scheme in Table I.

During the fractionation all solutions were kept at 0° and the centrifuging was carried out as rapidly as possible in pots initially cooled to below 0°. About 3 g. of freeze-dried Q3-fraction were obtained from 300 g. of bean flour. The phosphorylase preparations, from the same weight of flour, had an average total activity of 700 units.

Standard Digests for the Enzymic Hydrolysis of Polysaccharides at pH 7·0.—The polysaccharide (dry wt. *ca.* 200 mg.) was moistened with alcohol and dissolved in 0·012N-sodium hydroxide (50 c.c.) by gentle heating on a boiling-water bath, cooled, neutralised to phenolphthalein with dilute sulphuric acid, and diluted to 100 c.c. The concentration of the solution was now adjusted to 186·7 mg. of polysaccharide per 100 c.c. Of this solution a portion (7·5 c.c. ≡ 14 mg. of polysaccharide) was incorporated in a digest containing 0·2M-citrate buffer (pH 7·0; 3 c.c.), water (1·5 c.c.), and enzyme solution (2 c.c.). The digest was incubated at 21°, and at intervals portions (1 c.c. ≡ 1 mg. of polysaccharide) were removed for determination of B.V., and further portions (2—5 c.c.) for determination of reducing power. A control digest containing water in place of polysaccharide solution was incubated in parallel to determine the reducing power of the enzyme preparation.

Properties of the Bean Q-Enzyme. Action on Amylose.—(a) *Changes in B.V. and reducing power.* Q3-Fraction (0·6092 g. of freeze-dried powder) was dissolved in water (11 c.c.), and a portion (6 c.c.) incorporated in a standard digest containing three-fold quantities of all constituents. B.V. and reducing power were determined at intervals as described. The results are recorded in the following table and the % A.V.–% conversion curve is given in Fig. 1.

Action of Q3-fraction on amylose (B.V., 1·40).

Age of digest, mins.	0	30	240	270	362	480	510	630	1280
A.V.	1·40	1·05	0·540	0·505	0·421	0·337	0·320	0·260	0·104
Apparent conversion into maltose, %	0	0·0	—	4·1	—	—	8·1	10·0	18·2

(b) *β-Amylolysis of Q-enzyme-treated amylose.* Three digests were prepared containing amylose solution (B.V., 1·43; 10 c.c., containing 10 mg.), 0·2M-acetate buffer (pH 7·0; 3 c.c.), and Q-enzyme (Q3) solution (2 c.c., containing 111 mg. of freeze-dried powder). The solutions were incubated at 21° and then inactivated (by being heated at 100° for 10 minutes in the closed vessel) after periods of 3, 10½, and 21½ hours severally (see Table I). The blue value of each digest was determined immediately before inactivation. A control digest containing water (2 c.c.) in place of enzyme solution was incubated at the same time, and heated to 100° after 21½ hours. To each solution, after being cooled, were added N-acetic acid (0·35 c.c., to adjust the pH to 4·8), β-amylase solution (2 c.c.; 880 units; in 0·2M-acetate buffer, pH 4·8), and water to 25 c.c. These digests were incubated at 35·5° and the reducing powers of 5-c.c. portions estimated after 5 and 10 hours. Control experiments using amylose solution and an inactive Q-enzyme preparation had shown that coprecipitation of amylose with the protein did not occur

when the mixture was heated at 100°, nor was the degree of β-amylolysis of the amylose affected by this treatment.

Determination of pH and Temperature Optima of Bean Enzymes.—(a) *Optimum pH of Q-enzyme.* A Q3-precipitate was obtained from 100 g. of bean flour by the standard method and dissolved in water (25 c.c.). Portions (2 c.c.) of this solution were mixed with the appropriate buffer (2 c.c.), any precipitate being removed on a centrifuge, and the supernatant liquid (2 c.c.) incorporated in a standard digest, citrate buffer being replaced by a 0·13N-sodium acetate–sodium veronal buffer, the pH of which was adjustable by the addition of 0·1N-sulphuric acid. The digests were incubated at 21° and at intervals (60 and 120 mins.) portions (1 c.c.) were removed for determination of B.V. (see table below), the portions of the digests of alkaline pH being acidified before the addition of iodine solution.

Optimum pH of Q-enzyme.

pH of digest	4·13	4·98	5·66	6·88	7·80	8·57
B.V.* 60 mins.	1·31	1·28	1·09	0·94	0·96	1·10
B.V.* 120 mins.	1·28	1·21	0·955	0·750	0·723	0·805

* Initial B.V. = 1·38.

(b) *Optimum temperature of Q-enzyme action.* A Q3-solution was prepared as above and incorporated in standard digests which were incubated at various temperatures, portions (1 c.c.) being removed for determination of B.V. at 30 and 60 mins.:

Optimum temperature of Q-enzyme action.

Temp. of incubation	10·4°	20·0°	21·8°	25·2°	35·5°
B.V.* 30 mins.	0·955	0·880	0·885	0·895	1·00
B.V.* 60 mins.	0·841	0·753	0·766	0·790	0·910

* Initial B.V. = 1·16.

(c) *Optimum pH of phosphorylase synthetic activity.* Since dipotassium glucose-1 phosphate acts as a buffer of high pH, solutions (0·2M.) were adjusted to the desired pH values by addition of dilute sulphuric acid and then diluted to 0·1M-concentration. Portions (1 c.c.) of these solutions were incorporated in digests containing soluble starch (1%; 0·5 c.c.) as primer, veronal buffer (0·5 c.c.), and P3-solution (1·5 c.c.; activity 3 units). The digests were incubated at 35·5° for 12 minutes, the enzyme inactivated by the addition of trichloroacetic acid (10%; 5 c.c.) and, after centrifuging, the free phosphorus in 1-c.c. portions determined. From these values were subtracted the amounts of free phosphorus found in control digests to which the trichloroacetic acid had been added before the enzyme. The controls were not incubated at 35·5° but were centrifuged immediately on addition of acid and enzyme. The results are shown below:

Optimum pH of phosphorylase.

pH of digest	4·63	5·32	6·19	6·42	7·01	7·36	8·93
Phosphorus liberated *	0·00	0·707	0·704	0·567	0·490	0·449	0·247

* Difference between light-absorption values of digest and control.

(d) *Optimum temperature of phosphorylase.* The synthetic activity of a P5-fraction was determined by the standard method at various temperatures:

Optimum temperature of phosphorylase.

Temp. of incubation	16·5°	21·2°	35·5°	44·0°	58·0°
Phosphorus liberated *	0·168	0·227	0·337	0·334	0·032

* See preceding table.

(e) *Optimum pH of α-amylase.* This was determined by measuring at intervals the reducing power developed in the digests which were previously used for the determination of the optimum pH of Q-enzyme [see under (a)].

Optimum pH of α-amylase.

pH of digest	4·13	4·98	5·66	6·88	7·80	8·57
Reduction equiv. (c.c. 0·005N-$Na_2S_2O_3$) 24 hrs.	0·00	0·27	0·41	0·32	0·17	0·09
Reduction equiv. (c.c. 0·005N-$Na_2S_2O_3$) 48½ hrs.	0·00	0·18	1·18	1·37	0·42	0·07

Test for Maltase in Phosphorylase and Q-Enzyme.—Solutions of P5-fraction (4 c.c.; 9 units) and Q3-fraction (4 c.c.; 0·182 g.) were incubated separately with 0·2M-citrate buffer (pH 7·0; 6 c.c.) and maltose solution (18 c.c.; 7·1 mg.). The appropriate control digests were also prepared. No increase in reducing power occurred during 20 hours.

Action of P5-Fraction on Amylose and Glucose-1 Phosphate.—A P5-solution (2 c.c.; 2·4 units) was added to a standard amylose digest which was incubated at 21°. Measurements of A.V. were made at intervals. The results are recorded in Fig. 2.

A P5-solution (2 c.c.; 2·6 units) was incorporated in a digest containing 0·5M-citrate buffer (pH 6·0; 2 c.c.), water (1·5 c.c.), 0·1M-dipotassium glucose-1 phosphate (1 c.c.), and an achroic dextrin solution, prepared by acid hydrolysis of starch (0·5 c.c.; 0·1%). At intervals portions (0·5 c.c.) were removed for measurement of A.V. (Fig. 2), 0·5 c.c. of standard iodine solution being used in a total volume of 50 c.c.,

and for determination of free phosphorus the amounts of which (in 0·5 c.c.) were 0·063, 0·172, 0·172, 0·175, and 0·175, after 1¼, 9, 26½, 48¼, and 66½ hours, respectively.

Action of Salivary Amylase on Amylose and Amylopectin.—Freeze-dried salivary amylase (25 mg. ≡ 2 c.c. of saliva) was dissolved in water (100 c.c.) and portions (1·2 c.c.) were added to two digests, the first containing amylose (B.V., 1·45; 12·5 c.c.; 25 mg.) and the second, amylopectin (B.V., 0·158; 12·5 c.c.; 25 mg.). Both digests contained 0·2M-acetate buffer (pH 4·8; 3 c.c.) and water to 25 c.c. Incubation was carried out at 35° and at intervals portions (1 c.c.) were removed for determination of A.V. The results are given in Table II.

Action of Enzyme Preparations on Amylose and β-Dextrin.—Two standard digests were prepared, both containing the enzyme under examination; one contained amylose, and the other β-dextrin. The digests were incubated at 21°, and at equal periods after addition of the enzyme, portions (1 c.c.) were removed for determination of A.V. The amylose digest portion was stained under normal conditions but that from the β-dextrin digest was stained by using twice the normal concentration of polysaccharide and iodine in order to increase the accuracy of measurement. The results were plotted as % original A.V. of amylose against % original A.V. of β-dextrin, as in Fig. 3.

Further Purification of Phosphorylase and Q-Enzyme.—(a) *Reprecipitation with ammonium sulphate.* Details of the purification of phosphorylase by this method are given in Table I. A precipitated Q3-fraction was prepared from " Prolific Longpod " bean flour (100 g.) by the standard method and dissolved in water (50 c.c.). Portions (2 c.c.) of this solution were added to standard amylose and β-dextrin digests as above, and a Q4-fraction precipitated from the remainder of the solution by addition of ammonium sulphate to 19 g./100 c.c. The precipitate was dissolved in water (46 c.c.) and reprecipitated as above to give fraction Q5. This was dissolved in water (46 c.c.), and two portions (2 c.c. each) added to standard digests. The reprecipitation was continued in like manner to the Q9 stage, and test portions of the Q7 and Q9 fractions were similarly incubated with amylose and β-dextrin. Measurements of A.V. were made as previously described, and the results are plotted in Fig. 4.

(b) *Adsorption of enzymes on lead phosphate, cotton wool, cellulose, and starch.* The following general procedure was adopted. Four columns (19 mm. diam.) were prepared and packed to a depth of 12 cm. with (*a*) lead phosphate (prepared as by Peters, *J. Biol. Chem.*, 1908–9, **5**, 367) and powdered glass (filter aid), (*b*) whole potato starch and powdered glass, (*c*) fat-free cotton wool, and (*d*) powdered cellulose (from " Ashless filter tablets "). Columns (*c*) and (*d*) were perfused with soluble starch solution (0·1%; 100 c.c.) and washed until the effluent no longer stained with iodine. Freeze-dried P3-fraction was dissolved in water, precipitated by adding ammonium sulphate to 35 g./100 c.c., and the precipitate dissolved in water; the buffer used in freeze-drying was thereby removed. The phosphorylase activity of the solution was determined and portions were added to standard amylose and β-dextrin digests. The remaining solution was passed through the column under gravity, and the total effluent tested similarly. The lead phosphate adsorbed all enzymic activity, and the effluents from columns (*b*), (*c*), and (*d*) were unchanged in respect of relative phosphorylase and amylase activities.

The Q-Enzyme of the Wrinkled Pea.—An extract was prepared from wrinkled peas (Laxton's " Progress ") by shaking the ether-extracted flour (200 g.) with water (800 c.c.) for 30 minutes at room temperature. The centrifuged extract (495 c.c.) was dialysed against running distilled water for 24 hours at 2°, and the dialysed solution stirred with kaolin, which was then removed on a supercentrifuge. To the solution was added ammonium sulphate to a concentration of 19 g./100 c.c. After standing at 0° for 15 hours the precipitate (Q1) was removed, dissolved in water (50 c.c.), and reprecipitated twice as above, giving fraction Q4. The action of this fraction on amylose is shown in Fig. 1. It was free from maltase.

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