20. *The Enzymic Synthesis and Degradation of Starch. Part VI. The Properties of Purified P- and Q-Enzymes.*

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Directions are given for the preparation of the phosphorylase (P-enzyme) and the cross-linking enzyme (Q-enzyme) of potato as stable, freeze-dried powders. The freeze-drying must be carried out in a citrate buffer of pH 6—7, otherwise both enzymes are destroyed.

The purified P-enzyme (fraction P2) contained no maltase and only traces of Q-enzyme or the normal amylases. It showed a slight phosphatase activity on glycerophosphate but not on glucose-1 phosphate.

The purified Q-enzyme (fraction Q3) was substantially free from maltase, the amylases, and phosphatase. It contained a little P-enzyme, equivalent to 5% of that in the original juice.

Fraction P2 has *per se* no effect on the viscosity of starch paste. Q-Enzyme, on the other hand, has a striking liquefying action, in the presence or absence of phosphate.

Q-Enzyme has a strong accelerating effect on the rate of synthesis of polysaccharide from glucose-1 phosphate by P-enzyme. Q-Enzyme is irreversibly inactivated by being precipitated from aqueous solution by acetone.

The conversion of amylose into amylopectin by Q-enzyme is not inhibited by phloridzin. Moreover, the synthetic activity of P-enzyme is only slightly inhibited by phloridzin.

The significance of these observations is discussed in the succeeding paper (Part VII).

The study of the extraction and the properties of the cross-linking (Q-) enzyme of potato, described in Part IV of this series (Barker, Bourne, and Peat, *J.*, 1949, 1705), has been continued and extended to include the potato-phosphorylase (P-enzyme) which is separated in the same process. The method of isolation of the two enzymes is diagrammatically represented in Table I.

It was reported in Part IV that precipitates of Q-enzyme could be freeze-dried with little loss of activity if, to the moist precipitate, boiled potato juice were added before it was submitted to freeze-drying. It seemed probable that the boiled juice was simply buffering the enzyme slurry which, in its absence, became acid owing to loss of ammonia from ammonium sulphate. We have now been able to confirm this supposition and to show by the use of suitable buffers that P-enzyme preparations may also be freeze-dried without loss of activity.

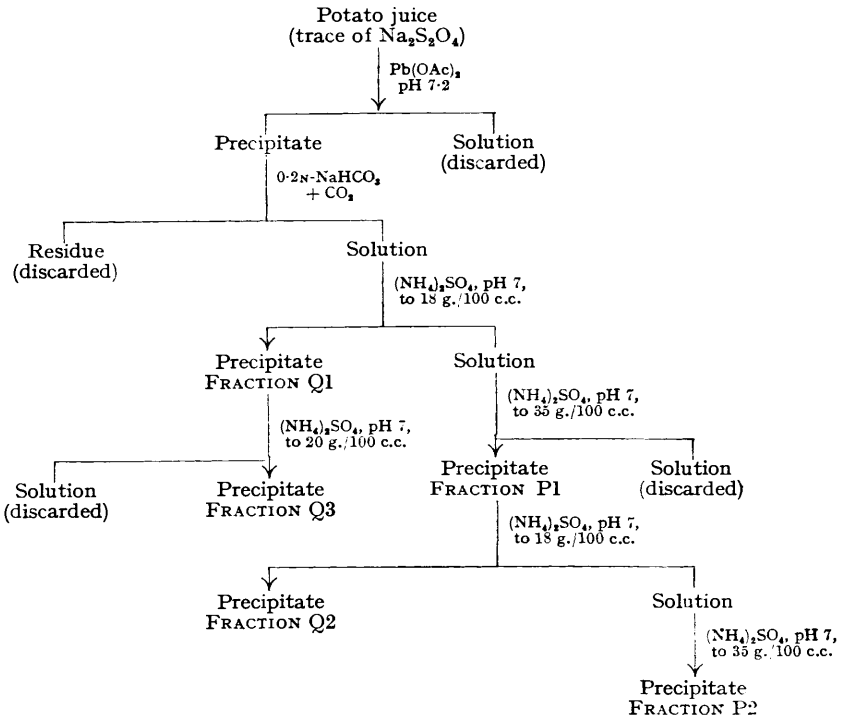
P-Enzyme.—The synthetic activity of fraction P2 (Table I) was completely destroyed by freeze-drying, whereas the less pure fraction, P1, retained about 25% of its activity. During the freeze-drying of the latter fraction the pH fell from 7·3 to *ca.* 5·5 (Table II). Boiled and filtered potato juice stabilised both fractions to freezing and the maximum acidity change was, in this case, from pH 7·2 to 6·4 (Table III). The effect of freeze-drying P1 (and P2) fractions in the presence of different buffers is shown in Tables IV and V. It was concluded that complete retention of the activity of P-enzyme was possible if the freeze-drying was carried out in a 0·2M-citrate buffer of pH 6·0.

Q-Enzyme.—It is shown in Table VI that the activity of Q-enzyme (in the conversion of amylose into amylopectin) is completely retained if a Q3 fraction (see Table I) is freeze-dried in a 0·2M-citrate buffer of pH 6·0, 6·5, or 7·0, and for routine preparations we have adopted the procedure of freeze-drying in such a citrate buffer at pH 7·0. It is a matter of considerable interest that, although a citrate buffer of pH 7·0 allows of maximum retention of Q-activity, yet an acetate buffer of the same pH is quite ineffective. In fact, a Q3 preparation freeze-dried in an acetate buffer of pH 7·0 was almost completely inactivated (Table VI). This loss of activity appears to be associated with the development of acidity during the drying process. The final pH with acetate was 5·15, as compared with 6·5 when citrate was used. Table IV indicates that P-enzyme also is destroyed by freeze-drying in an acetate buffer of pH 7·0.

Stocks of fractions P2 and Q3 were prepared from more than 60 kg. of potatoes during the "resting" stage, *i.e.*, when the amylase content was a minimum, and were freeze-dried in citrate buffers of pH 6·0 and 7·0, respectively. The dry powders were stable during prolonged periods; for instance, the dried fraction P2 lost not more than 1% of its activity in 60 days,

TABLE I.

Isolation of P- and Q-enzymes from potato juice.



whereas the activity of the same sample kept in solution in citrate buffer (pH 6·0) for the same time fell to one-tenth of its initial value. The Q3 powder showed no change in Q-enzyme activity in 41 days.

The Enzymic Purity of Fractions P2 and Q3.—The Q3 fraction was shown in Part IV to be substantially free from the normal amylases and from maltase. It contained a little P-enzyme (detected by its synthetic activity) corresponding, after freeze-drying, to about 5% of the P-enzyme initially present in the juice. The P2 fraction was also shown to be free from maltase (lack of maltose-hydrolysing activity). It contained, at most, only traces of Q-enzyme and the normal amylases inasmuch as it effected, at pH 6—7, only a very slow fall in the A.V. (6800 A.) of amylose.

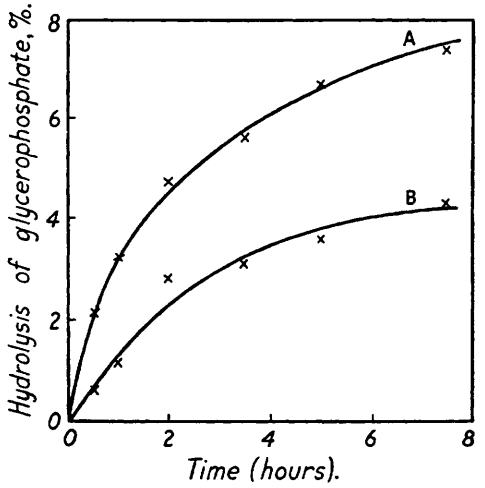
Phosphatase Content.—The Q3 fraction did not liberate a detectable amount of free phosphate from sodium glycerophosphate in 7·5 hours at pH 6·5 and might thus be considered as free from phosphatase. The freeze-dried P2 fraction did, however, contain a trace of phosphatase since it effected a 7·5% conversion of glycerophosphate into free phosphate in 7·5 hours (see Fig. 1, curve *A*). It appeared that this reaction was either reversible or was inhibited by phosphate ion since the hydrolysis of glycerophosphate proceeded much more slowly in the presence of added mineral phosphate (Fig. 1, curve B). Fraction P2 exerted no phosphatase activity with respect to glucose-1 phosphate under the normal conditions of polysaccharide synthesis. Thus, when fraction P2 was incubated at pH 6·0 in the presence of starch with glucose-1 phosphate no development of reducing power occurred in one hour.

Starch-liquefying Action of P- and Q-Enzymes.—The relative viscosity of a 1% dispersion of potato starch in acetate buffer (pH 7·0) was not affected by fraction P2. In a phosphate buffer of pH 6·7, a slow fall in the viscosity was observed (Fig. 2) and could be attributed to normal phosphorolysis of the starch.

It was reported in Part I (Bourne and Peat, *J.*, 1945, 877) that the crude preparations of the branching enzyme then available liquefied starch pastes. That this is an intrinsic action of Q-enzyme has now been demonstrated by the striking diminution of viscosity of a 1% starch paste brought about by the highly purified Q3 fraction (Fig. 2). As pointed out above,

FIG. 1.

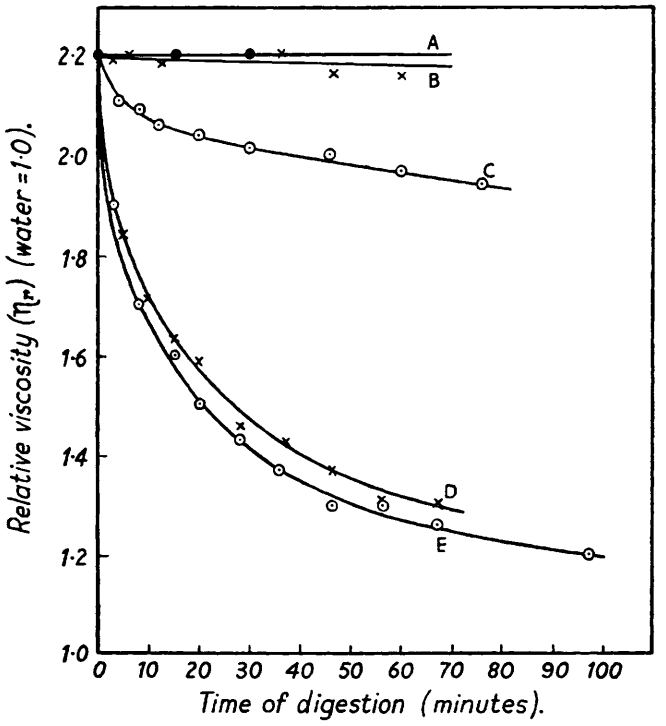
Phosphatase activity of enzyme fraction P2 *(sodium glycerophosphate substrate).*



A. Mineral phosphate not added to digest.
B. Mineral phosphate added to digest.

FIG. 2.

The action of fractions P2 *and* Q3 *on the viscosity of starch paste in the presence and absence of mineral phosphate.*



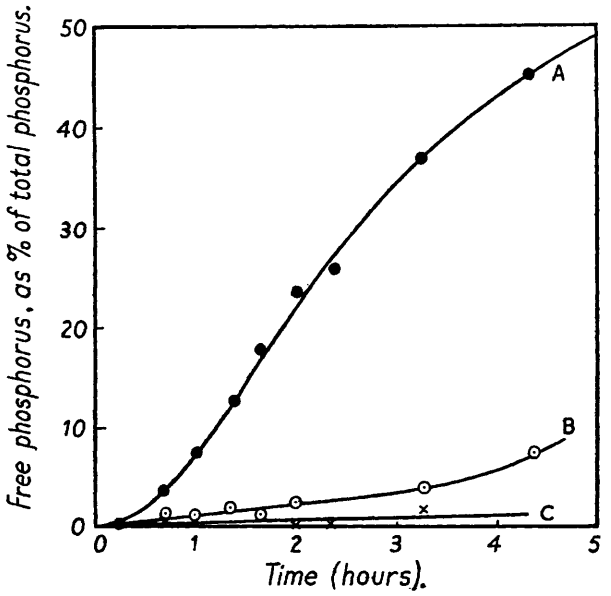
A. Water (acetate buffer).
B. P_2 *(Acetate buffer).*
C. P_2 *(Phosphate buffer).*
D. Q_3 *(Acetate buffer).*
E. Q_3 *(Phosphate buffer).*

there are reasons for believing that fraction Q3 does not contain α-amylase in sufficient concentration to cause this degree of liquefaction. Nor can the liquefaction be ascribed to phosphorolysis. It is true that a somewhat greater fall in viscosity is registered if mineral phosphate is present in the Q3 digest (Fig. 2), but we ascribe this enhanced effect in the presence of phosphate to the small proportion of P-enzyme which the Q3 fraction contains.

Autocatalysis in Polysaccharide Syntheses from Glucose-1 Phosphate.—The rate of liberation of free phosphate by P-enzyme acting on glucose-1 phosphate is a measure of the rate of synthesis of polysaccharide, and Fig. 3 shows that this rate is greatly accelerated if Q-enzyme is also present. The enzyme preparations used in this experiment were purified beyond the stages given in Table I, by two further precipitations with ammonium sulphate, giving fractions P4 and Q5. Curve *C* in Fig. 3 shows that the P-enzyme content of fraction Q5 is negligibly small. The autocatalytic effect is shown by the difference between curve *B* (P4 fraction alone)

FIG. 3.

Autocatalysis in polysaccharide synthesis from glucose-1 phosphate.



A. P-*Enzyme* + Q-*enzyme*.
B. P-*Enzyme alone*.
C. Q-*Enzyme alone*.

and curve *A* (P4 and Q5 acting together). Cori and Cori (*J. Biol. Chem.*, 1943, **151**, 57) showed that the " branching factor " of liver and heart had a similar catalytic effect on the synthetic action of muscle phosphorylase, and ascribed the effect to the increase in the proportion of non-reducing chain ends brought about by the branching factor. Clearly Q-enzyme functions in the same way. It has been indicated by Bernfeld and Meutémédian (*Helv. Chim. Acta*, 1948, **31**, 1724) that α-amylase, which fragments amylose chains, would, by increasing the number of chain ends, also catalyse the synthesis from glucose-1 phosphate (see also Part III, Bourne, Peat, and Sitch, this vol., p. 1448). This autocatalytic action cannot therefore be regarded as a specific test for Q-enzyme.

The Inactivation of Q-Enzyme by Acetone.—It was shown in Part II (Bourne, Macey, and Peat, *J.*, 1945, 882) that Q-enzyme was inactivated by precipitation from aqueous solution with ethyl alcohol (3 vols.) whereas salivary α-amylase was unaffected by alcohol. We have now found that the activity of the purified Q-enzyme (fraction Q3) is almost completely destroyed when it is precipitated from a citrate buffer solution at pH 6 and 0° with acetone at concentrations between 25 and 40%.

The Effect of Phloridzin on P- and Q-Enzymes.—Green and Stumpf (*J. Biol. Chem.*, 1942 **142**, 355) reported that potato phosphorylase was partly inhibited (in its synthetic action) by phloridzin. At a phloridzin concentration of (approx.) $1{\cdot}4 \times 10^{-3}$M., the inhibition was only

18%, and at half this concentration there was no inhibition of phosphorylase. Bernfeld and Meutémédian (*loc. cit.*, p. 1735) showed that isophosphorylase was not inhibited by phloridzin and, on the assumption that phosphorylase was strongly inhibited by phloridzin, added the latter reagent to mixtures of phosphorylase and isophosphorylase in order to influence the relative active concentrations of the two enzymes.

We have now examined the effect of phloridzin on the conversion of amylose into amylopectin by Q-enzyme and on the synthesis of amylose from glucose-1 phosphate by P-enzyme. Caution had to be observed when measuring the activity of fraction Q3 by diminution of A.V. (6800 A.) for it was shown that phloridzin reacts rapidly with free iodine and also, but more slowly, changes the blue colour of an amylose–iodine complex. To overcome this difficulty when Q-enzyme was incubated with amylose in the presence of phloridzin, more than the standard concentration of iodine was employed and the absorption value was measured immediately after staining. By adopting this technique we were able to show that Q3 fraction was not inhibited at all by $1{\cdot}5 \times 10^{-3}$M-phloridzin (see Table VII).

Furthermore, we found that the introduction of phloridzin ($1{\cdot}24 \times 10^{-3}$M.) into a digest of glucose-1 phosphate and P-enzyme (fraction P2) diminished the enzyme activity by only 8% (Table VIII).

Experimental.

Analytical Methods.—The analytical methods employed were described in Part IV (Barker, Bourne, and Peat, *J.*, 1949, 1705).

Fractionation of Starch.—The amylose samples were isolated from potato starch by precipitation with thymol (Bourne, Donnison, Haworth, and Peat, *J.*, 1948, 1687).

Isolation of P- and Q-Enzymes from Potatoes.—The juice (100 c.c.) from King Edward potatoes was treated with lead acetate and centrifuged. The lead–protein complex was eluted by stirring with sodium hydrogen carbonate in the presence of a stream of carbon dioxide (details of these early stages of the isolation were given in Part IV). To the eluate (*ca.* 110 c.c.) ammonium sulphate solution (pH 7; 50 g./100 c.c.) was added until the concentration of this salt reached 18 g./100 c.c. The precipitate (fraction Q1) was separated by the centrifuge. The concentration of ammonium sulphate in the supernatant liquid was increased to 35 g./100 c.c. and the precipitate (fraction P1) was collected.

Fractionation of P1. Fraction P1 was dissolved in water (20 c.c.) and ammonium sulphate solution (pH 7·0; 50 g./100 c.c.) was added until the salt concentration was 18 g./100 c.c. The precipitate (fraction Q2) was removed and the concentration of ammonium sulphate in the supernatant liquid was increased to 35 g./100 c.c. The precipitate (fraction P2) was collected by the centrifuge.

Fractionation of Q1. Fraction Q1 was dissolved in water (20 c.c.) and kept at 0° for 1 hour. A small insoluble residue was removed before the concentration of ammonium sulphate in the solution was adjusted, as above, to 20 g./100 c.c. The precipitated fraction (Q3) was separated by the centrifuge.

Except where otherwise stated, the operations were carried out at room temperature.

Freeze-drying of P-Enzyme Solutions.—The enzyme solutions were frozen in an alcohol–carbon dioxide mixture and dried at 0·01 mm. No thawing occurred under these conditions. The activities of the enzyme samples were measured by the method of Green and Stumpf (*loc. cit.*).

(a) *Freeze-drying of fractions P1 and P2.* The enzyme fraction (P1 and P2) isolated from 100 c.c. of juice was dissolved in water, and the volume adjusted to 20 c.c. Half of the solution was freeze-dried, the powdery product being redissolved in water and diluted to 10 c.c. The other half served as a control. The results obtained with several different samples of P1 and P2 are given in Table II.

Table II.

Freeze-drying of fractions P1 and P2.

Enzyme fraction.	pH of enzyme solution: before drying.	pH of enzyme solution: after drying.	Retention of P-enzyme activity during freeze-drying (%).	Enzyme fraction.	pH of enzyme solution: before drying.	pH of enzyme solution: after drying.	Retention of P-enzyme activity during freeze-drying (%).
P1	7·20	5·65	45·7	P1	7·35	5·06	8·6
,,	7·34	5·65	15·8	,,	7·29	5·60	17·7
,,	7·36	5·51	42·6	P2	—	—	0·0
,,	7·24	5·71	35·0	,,	—	—	0·0

(b) *Freeze-drying of fractions P1 and P2 in the presence of boiled potato juice.* A sample of potato juice was boiled for 20 minutes, filtered, and diluted to its original volume with distilled water. To a solution (20 c.c.) of fraction P1 or P2, obtained from 100 c.c. of juice, 5 c.c. of the boiled juice were added and the enzyme solution thus obtained was divided into two equal portions. One of these was freeze-dried, the dry residue being redissolved in water and diluted to 12·5 c.c. The second half served as a control. It was essential that this control should contain boiled juice because, under these conditions, the addition of boiled juice to fraction P1 or P2 increased the activity of the phosphorylase solution by about 15%. The stabilising effect of boiled juice during the freeze-drying of P-enzyme solutions is shown in Table III.

(c) *Freeze-drying of fraction P1 in the presence of different buffers.* The boiled juice used in (b) was replaced by different buffer solutions as shown in the Table IV. It was observed that the mere addition of 0·2M-sodium hydrogen carbonate, even without freeze-drying, diminished the P-enzyme activity by about 30%.

TABLE III.

Freeze-drying of fractions P1 and P2 in the presence of boiled potato juice.

Enzyme fraction.	pH of enzyme solution, containing boiled juice: before drying.	after drying.	Retention of P-enzyme activity during freeze-drying (%).
P1	7·09	6·40	95·0
,,	7·26	6·40	100·0
P2	—	—	100·0

TABLE IV.

Freeze-drying of fraction P1 in the presence of different buffers.

Buffer solution.	Volume of buffer added to 20 c.c. of P1 solution (c.c.).	Retention of P-enzyme activity during freeze-drying (%).
0·1M-Citrate (pH 6·3)	4·0	66·8
0·1M-Glycine (pH 8·2)	,,	59·5
1·0M-Acetate (pH 7·0)	,,	0·0
0·2M-$NaHCO_3$	,,	47·2
,,	,,	53·0
,,	,,	51·8
0·5M-$NaHCO_3$	8·0	29·4
,,	,,	51·5

(d) *Freeze-drying of solutions of fractions P1 and P2 in 0·2M-citrate buffer (pH 6·0).* Fraction P1 (or P2), isolated from 100 c.c. of potato juice, was dissolved directly in 0·2M-citrate buffer (pH 6·0) to give 20 c.c. of enzyme solution, half of which served as a control. The other half was freeze-dried, the product being redissolved in water and diluted to 10 c.c. The efficacy of the citrate buffer as a stabiliser during the freeze-drying of P1 and P2 fractions is shown in Table V, where the results obtained with several different samples of these enzymes are recorded.

TABLE V.

Freeze-drying of solutions of fractions P1 and P2 in 0·2M-citrate buffer (pH 6·0).

Enzyme fraction	P1	P1	P1	P1	P2	P2
Retention of P-enzyme activity during freeze-drying (%)	100·0	98·5	100·0	100·0	100·0	100·0

In view of the above results, this method of freeze-drying fractions P1 and P2 in 0·2M-citrate buffer (pH 6·0; 20 c.c. for each 100 c.c. of juice originally used) was adopted as the standard method for the preparation of the enzymes in powder form, and has been successfully employed for the isolation and storage of the P-enzyme from more than 60 kg. of potatoes.

Freeze-drying of Fraction Q3 in the Presence of Citrate and Acetate Buffers.—Fraction Q3, isolated from 125 c.c. of juice, was dissolved in 0·2M-citrate buffer (pH 6·0; 25 c.c.). A portion (20 c.c.) of this solution was freeze-dried, as above, the rest being retained as a control. The dry powder was dissolved in water, diluted to 20 c.c., and incorporated in a digest consisting of amylose solution (14 mg. in 7·5 c.c., prepared as in Part IV), maltose solution (3·0 mg. in 1·5 c.c.), 0·2M-citrate buffer (pH 6·0; 3 c.c.), and the buffered enzyme solution (2·0 c.c.), making a total volume of 14·0 c.c. A second digest contained the enzyme control. After 30 minutes at 20·5° an aliquot portion (1·0 c.c.) of each digest was stained with iodine (2·0 mg.)–potassium iodide (20·0 mg.) in a final volume of 100 c.c. and the A.V. (6800 A.) was measured in the usual manner.

In three similar experiments the 0·2M-citrate buffer (pH 6·0) was replaced, both during freeze-drying and in the digests, by other buffers, namely, 0·2M-citrate buffer (pH 6·5), 0·2M-citrate buffer (pH 7·0), and 0·2M-acetate buffer (pH 7·0). The results are shown in Table VI.

TABLE VI.

Freeze-drying of solutions of fraction Q3 in acetate and citrate buffers.

Buffer used during freeze-drying and in the digests.	Fall (%) in A.V. (6800 A.) in 30 mins. Control.	After freeze-drying.	pH of enzyme-buffer solution after freeze-drying.
0·2M-Citrate (pH 6·0)	74·7	72·6	5·83
0·2M-Citrate (pH 6·5)	79·4	76·8	6·21
0·2M-Citrate (pH 7·0)	79·5	76·8	6·51
0·2M-Acetate (pH 7·0)	74·5	0·8	5·15

For routine purposes, the standard technique adopted for the freeze-drying of fraction Q3 was to dissolve the enzyme in 0·2M-citrate buffer (pH 7·0; 12·5 c.c. for each 100 c.c. of juice), freeze the solution in an alcohol–carbon dioxide mixture, and dry it at 0·01 mm. In this way a very active enzyme preparation has been obtained in powder form from more than 60 kg. of potatoes.

The Stability of Fractions P1 and P2 when stored at 0°.—Fraction P1 was dissolved in 0·2M-citrate buffer (pH 6·0; 20 c.c. for each 100 c.c. of juice originally employed) and the solution was divided into two parts. One of these was freeze-dried as above, the other being kept as a control. The solution and the dry powder were stored at 0° in tightly-stoppered bottles, and the P-enzyme activity of each was measured at intervals by the method of Green and Stumpf (*loc. cit.*). In a second experiment fraction P2 was used. The rates of decay in P-enzyme activity are shown below.

The stabilities of fractions P1 and P2 in citrate buffer (pH 6·0) at 0°.

Enzyme fraction.	Method of storage.	Retention of P-enzyme activity (%). 12 days.	27 days.	60 days.
P1	Solution	94·2	83·5	60·0
,,	Powder	100·0	99·0	98·0
P2	Solution	59·0	49·5	9·1
,,	Powder	100·0	100·0	99·0

The Stability of Fraction Q3 when stored at 0° as a Powder.—Fraction Q3 was dissolved in 0·2M-citrate buffer (pH 7·0) and freeze-dried according to the standard technique described above. The powder was stored at 0° in a well-stoppered bottle, samples being periodically removed and incubated with amylose in the manner already described. The figures quoted in the following table show that there was no detectable loss in Q-enzyme activity in 41 days.

The stability of fraction Q3 when stored at 0° in powder form.

Time of storage (days)	10	27	41
Fall (%) in A.V. (6800 A.) of amylose in 30 mins.	75·0	75·2	75·1

Tests for Phosphatase in Fractions P2 and Q3.—The enzyme fractions (P2 and Q3) used in these tests had been stored for several weeks as the dry powders obtained when their solutions in 0·2M-citrate buffers were freeze-dried under the standard conditions already described.

(a) *With sodium glycerophosphate as substrate for fraction P2.* The solutions used were 0·1M-sodium glycerophosphate in 0·2M-citrate buffer (pH 6·5), potassium dihydrogen phosphate in water (*ca.* 3·2 mg. phosphorus/c.c.), and fraction P2 in water (40 c.c. for each 100 c.c. of juice employed for the isolation of the enzyme). These solutions were incorporated in four digests (A—D), the compositions of which are shown in section (i) of the following table. The digests were incubated at 20° and free-phosphate estimations were made periodically; these are recorded in section (ii).

Action of fraction P2 on sodium glycerophosphate.

(i) *Compositions of digests.*

Solution.	Digest, c.c. A.	B.	C.	D.
Sodium glycerophosphate	10	10	10	10
Fraction P2	4	4	—	—
Potassium dihydrogen phosphate	1	—	1	—
Water	—	1	4	5

(ii) *Free phosphate estimations.*

Time of incubation (hours).	Free phosphorus (mg. per 15 c.c. digest). A.	B.	C.	D.
0·5	3·420	0·673	3·210	0·000
1·0	3·570	1·020	—	—
2·0	4·080	1·485	—	—
3·5	4·170	1·760	—	—
5·0	4·350	2·100	—	—
7·5	4·560	2·310	3·210	—

The inorganic phosphate liberated by enzyme action was measured (i) in the presence of potassium dihydrogen phosphate by the difference between digest (A) and (C), and (ii) in the absence of potassium dihydrogen phosphate by the difference between digest (B) and (D). These values are plotted in Fig. 1.

(b) *With sodium glycerophosphate as substrate for fraction Q3.* The above experiment was repeated using a solution of fraction Q3 in water (40 c.c. for each 100 c.c. of juice employed for the isolation of the enzyme) instead of the solution of fraction P2. No mineral phosphate was liberated by enzyme action in digest (B) in 7·5 hours, showing the absence of phosphatase activity in fraction Q3.

(c) *With dipotassium glucose-1 phosphate as substrate for fraction P2.* A digest containing the solution of fraction P2 (1·5 c.c.) used in (a), 0·1M-dipotassium glucose-1 phosphate in 0·2M-citrate buffer (pH 6·0; 1·0 c.c.), 0·5M-citrate buffer (pH 6·0; 0·5 c.c.), and starch solution (0·5 c.c., containing 0·5 mg. of starch) was incubated at 20·5° for 1 hour. There was no increase in reducing power, thus proving the absence, under these conditions, of phosphatase action on the glucose-1 phosphate.

The Influence of Fractions P2 and Q3 on the Viscosities of Starch Pastes in the Absence and in the Presence of Mineral Phosphate.—Enzyme solutions. The P2 and Q3 enzyme fractions, which had been

stored at 0° for several weeks after being freeze-dried by the standard methods given above, were each dissolved in water (20 c.c. for each 100 c.c. of juice used for the isolation of the enzymes). The P2 solution contained 14·1 units of phosphorylase/c.c., and the Q3 solution 0·8 unit of phosphorylase/c.c. In a preliminary experiment it was shown that this Q3 solution effected a maximum diminution of 83% in the A.V. (6800 A.) of amylose (B.V., 1·04) with only 2·4% "apparent conversion into maltose," a fact which established the absence of more than traces of the normal amylases from the enzyme sample (see Barker, Bourne, and Peat, Part IV, *loc. cit.*).

Starch pastes. Potato starch (dry weight, 5 g.) was dispersed in boiling water containing sodium chloride (0·5 g.). The solution was cooled and diluted to 450 c.c. with water. To half of this solution 0·2M-acetate buffer (pH 7·0; 25 c.c.) was added, giving solution (A). To the other half 0·2M-phosphate buffer (pH 6·7; 25 c.c.) was added, giving solution (B). The concentrations of starch and buffer in both (A) and (B) were 1% and 0·02M., respectively.

Viscosity measurements. The starch solution (10 c.c.; A or B) and enzyme solution (1 c.c.; P2 or Q3) were adjusted to 20°, mixed, and introduced into an Ostwald viscometer, which was immersed in a thermostat at 20°. Viscosity measurements were made at intervals and are plotted in Fig. 2. In a second series of experiments, in which the volumes of the two buffers employed were increased from 25 c.c. to 75 c.c., similar results were obtained.

The Effect of Phloridzin on the Amylose–Iodine Stain.—Amylose was stained with iodine under the standard conditions for the estimation of B.V. (Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924). The coloured solution contained amylose (1 mg./100 c.c.), iodine (2 mg./100 c.c.), and potassium iodide (20 mg./100 c.c.). A second portion of amylose was stained in the same way except that the final coloured solution contained, in addition, phloridzin (to $3{\cdot}33 \times 10^{-5}$M.). The absorption values, measured immediately after the preparation of the solutions, are shown below. The amylose–iodine

The effect of phloridzin on the amylose–iodine stain.

Phloridzin concentration.	Absorption value at λ (in A.): 4300.	4700.	4900.	5200.	5500.	5800.	6000.	6800.
$3{\cdot}33 \times 10^{-6}$M.	0·40	0·42	0·44	0·51	0·71	0·94	1·04	1·08
Nil	0·63	0·54	0·54	0·59	0·75	0·97	1·07	1·11

stain slowly faded in the presence of phloridzin, indicating that the initial rapid reaction of this compound with the excess of iodine (revealed in the relative absorption values) was followed by a slower reaction with the iodine involved in complex formation with the amylose.

The Effect of Phloridzin on the Conversion of Amylose by Fraction Q3.—Enzyme fraction Q3 was incubated with amylose (B.V., 1·30) under the conditions of the standard digest described by Barker, Bourne, and Peat (Part IV, *loc. cit.*). A second digest included the same components together with phloridzin ($1{\cdot}5 \times 10^{-3}$M.). The course of each reaction was followed by staining, at intervals, an aliquot portion (1 c.c. ≡ 1 mg. of amylose) of the digest with iodine (6 mg.) and potassium iodide (60 mg.) in a total volume of 100 c.c. The absorption values of the stained solutions, which were measured as soon as possible after staining, are recorded in Table VII, from which it is seen that phloridzin does not inhibit the action of Q-enzyme on amylose under the conditions of the experiment.

TABLE VII.

The effect of phloridzin on the conversion of amylose (B.V., 1·30) by fraction Q3.

Concentration of phloridzin in digest.	A.V. (6800 A.) after: 15 mins.	30 mins.	63 mins.
0	0·602	0·473	0·368
$1{\cdot}5 \times 10^{-3}$M.	0·602	0·467	0·352

Inhibition of P-Enzyme by Phloridzin.—(a) *Inhibition in the digest.* Fraction P2, which had been stored at 0° as the powder obtained when a solution in citrate buffer was freeze-dried by the standard technique described above, was dissolved in water (13·5 c.c. for each 100 c.c. of juice used in the preparation of the enzyme). This solution was incorporated in a digest which contained P2 solution (0·5 c.c.), 1% soluble starch solution (0·5 c.c.), 0·1M-glucose-1 phosphate in 0·2M-citrate buffer (pH 6·0; 1·0 c.c.), and water (1·5 c.c.). The digest was incubated at 35° for 12 minutes, and the reaction was then arrested by addition of 5% trichloroacetic acid solution (5·0 c.c.). After centrifuging, the free phosphate in the supernatant liquid was determined colorimetrically. The free phosphate originally present in the digest was determined by adding the trichloroacetic acid to the digest before the P2 solution was added. The mineral phosphate liberated by enzyme action was calculated by difference.

In other digests, the water (1·5 c.c.) was replaced by phloridzin solutions (1·5 c.c.) of different concentrations (0 to $2{\cdot}9 \times 10^{-3}$M.), and, by comparison with the above control, the effect of phloridzin on P-enzyme activity was determined; the results are given in Table VIII. Phloridzin does not interfere with the estimation of free phosphate.

TABLE VIII.

Inhibition of P-enzyme by phloridzin present in the digest.

Concentration of phloridzin in digest (M. × 10^{-3}) ...	0·00	0·44	0·89	1·06	1·24
Inhibition (%) of P-enzyme activity	0·0	2·2	6·6	8·1	8·1

When the solution of fraction P2 was diluted ten-fold and the concentration of phloridzin in the digest was increased to $2{\cdot}67 \times 10^{-3}$M., there was 40·5% inhibition of P-enzyme activity.

(b) *Pretreatment of fraction P2 with phloridzin.* The experiment (a) was repeated with one variation. Fraction P2 was dissolved directly in phloridzin solutions of different concentrations (40 c.c. for each 100 c.c. of juice used in the preparation of the enzyme) and kept at room temperature for 1 hour. A portion (1·5 c.c.) of each solution was added, together with water (0·5 c.c.), to the other components of the digest and the experiment was continued as above, giving the results recorded below.

Inhibition of P-enzyme by pretreatment with phloridzin.

Concentration of phloridzin (M. $\times 10^{-3}$).		Inhibition (%) of P-enzyme activity.	Concentration of phloridzin (M. $\times 10^{-3}$).		Inhibition (%) of P-enzyme activity.
During pretreatment of the enzyme.	In the digest.		During pretreatment of the enzyme.	In the digest.	
0·00	0·00	0·0	4·14	1·78	14·0
2·07	0·89	7·4	6·20	2·67	21·3
3·10	1·33	10·3			

Precipitation of Fraction Q3 with Acetone.—The following operations were performed at 0° in a cold room. Fraction Q3, which had been freeze-dried in citrate buffer in the usual way and stored at 0° for 7 weeks, was dissolved in water (40 c.c. for each 100 c.c. of juice used in the isolation of the enzyme). Portions (2·0 c.c.) of this solution were mixed with aqueous acetone solutions (4·0 c.c.) containing different concentrations of acetone. Each precipitate was collected by the centrifuge and stirred with a mixture of 0·2M-citrate buffer (pH 7·0; 2·0 c.c.) and 0·2M-sodium hydrogen carbonate solution (1·0 c.c.), the insoluble residue being removed by the centrifuge. An aliquot portion (2·0 c.c.) of each supernatant liquid was incubated at 20·5° with amylose (14 mg.; B.V., 1·04), maltose (3 mg.), and 0·2M-citrate buffer (pH 7·0; 2·5 c.c.) in a total volume of 14 c.c. As a control a portion (2·0 c.c.) of the original Q3 solution was mixed with 0·2M-sodium hydrogen carbonate solution (1·0 c.c.) and an aliquot portion (2·0 c.c.) of the mixture was incubated with amylose under the same conditions. After 30 minutes a sample (1·0 c.c.) of each digest was stained with iodine (2 mg.)–potassium iodide (20 mg.) in a total volume of 100 c.c. The values for A.V. (6800 A.) thus obtained are tabulated below.

The precipitation of fraction Q3 with acetone at 0°.

Conc. of acetone (% by vol.)	Control	26·6	33·0	40·0
Fall (%) in A.V. (6800 A.) of amylose in 30 mins.	72·5	8·5	6·7	4·1

Autocatalysis in Polysaccharide Synthesis by P- and Q-Enzymes.—Fraction P2 was further purified by two precipitations with neutral ammonium sulphate, the fraction precipitated between ammonium sulphate concentrations of 19 and 34 g./100 c.c. being collected each time. The product was dissolved in 0·2M-citrate buffer (pH 6·0; 20 c.c. for each 100 c.c. of potato juice used in the isolation of the enzyme) and freeze-dried. When required, the powder was dissolved in water (50 c.c. for each 100 c.c. of juice employed), giving a solution of P-enzyme which contained 2·5 units (Green and Stumpf) of phosphorylase per c.c.

Fraction Q3 was further purified by precipitating it twice with ammonium sulphate (18 g./100 c.c.) in the usual manner. The product was dissolved in 0·2M-citrate buffer (pH 6·5; 13 c.c. for each 100 c.c. of juice used for the isolation of the enzyme) and freeze-dried. When required, the powder was dissolved in water (20 c.c. for each 100 c.c. of juice employed), giving a solution of Q-enzyme which possessed 65% of the Q-enzyme activity present in the original Q3 fraction (measured by incubation with amylose and comparison with dilution curves for Q3). The solution of the purified Q-enzyme contained only 0·05 unit (Green and Stumpf) of phosphorylase per c.c.

These solutions of the purified enzymes were incorporated in digests which contained P-enzyme solution (6·75 c.c.) and/or Q-enzyme solution (6·75 c.c.), together with 0·1M-dipotassium glucose-1 phosphate (9·0 c.c.) and sufficient 0·2M-citrate buffer (pH 6·5) to make the total volume 31·5 c.c. The digests were incubated at 20° and free phosphate estimations were made at intervals, as shown in Fig. 3.

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