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The Inhibition of Plant Phosphorylases by β -Amylase and the Detection of Phosphorylase in Barley

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The enzyme phosphorylase may readily be detected in aqueous extracts of a number of starch-storing organs by the iodine-staining properties of the polysaccharide produced by its action on glucose-1-phosphate (Hanes, 1940*a, b*). In extracts of ungerminated cereal grains (wheat, oats, rye and barley), however, no such iodine-staining material arises under similar conditions. It appeared possible that the high concentration of β -amylase in cereal extracts might lead to β -amylolysis, either of the product of phosphorylase action on glucose-1-phosphate, or of the carbohydrate primer necessary to initiate synthesis. In such circumstances the absence of iodine-staining material does not preclude the presence of phosphorylase. Release of free phosphate would also be prevented if synthesis is inhibited by destruction of the primer, but if only the product of synthesis is destroyed then phosphate release should be observed. Accordingly, the fate of glucose-1-phosphate has been examined in more detail, and the effect of β -amylase on phosphorylase action studied, using preparations from the broad bean (*Vicia faba*) and the potato (*Solanum tuberosum*) as test systems. These investigations show that β -amylase inhibits phosphorylase action, but by some mechanism other than primer destruction, and further that phosphorylase is present in extracts of ungerminated barley. A preliminary communication on these experiments has been made (Porter, 1949).

EXPERIMENTAL

Substrates

Glucose-1-phosphate was prepared as the dipotassium salt by the method of Hanes (1940*b*), modified according to a Practical Class Sheet from Dr R. Hill, Biochemical

Laboratory, Cambridge. The twice recrystallized product (40 g.) was then dissolved in 500 ml. of water, the solution passed through cation and anion exchange columns (McCready & Hassid, 1944), and the ester recovered from the final effluent and again recrystallized. This additional procedure served to remove small traces of carbohydrate impurity which acted as primer in the phosphorylase reaction.

Amylose, prepared from potato starch by the thymol method (Bourne, Donnison, Haworth & Peat, 1948), was supplied by Dr W. J. Whelan.

Primers for the phosphorylase reaction

Soluble starch was prepared from commercial potato starch by the method of Hanes & Cattle (1938), and used in 1% (w/v) solution.

A solution of *achroic dextrins* was made by heating 1 g. of soluble starch with 25 ml. of 5*N*-H₂SO₄ in a boiling-water bath until the mixture just failed to give a colour reaction with I₂ (about 15 min.). The cooled solution was neutralized with 5*N*-NaOH and diluted to 100 ml.

Amylose glycollate, supplied by Dr W. J. Whelan, was prepared as described by Peat, Bourne & Thrower (1947) and then exhaustively treated with soya-bean β -amylase.

Enzymes

Potato phosphorylase I was prepared by the method of Hanes (1940*b*). The final precipitate at 50% saturation with (NH₄)₂SO₄ was taken up in 25 ml. of 0.25*M*-citrate at pH 6.8, and the solution stored in the cold. The preparation was self-priming with respect to polysaccharide synthesis after a time lag of about 45 min. and contained a little α -amylase.

Potato phosphorylase II and broad-bean phosphorylase were prepared by the Pb precipitation technique (Barker, Bourne & Peat, 1949) and supplied by Mr P. Hobson in the form of freeze-dried powders. With these two preparations the time lag before the onset of polysaccharide synthesis, in the absence of added primer, was much longer than with potato phosphorylase I, and only traces of α -amylase were present.

Crystalline β -amylase from the sweet potato was the preparation of Dr A. K. Balls.

Barley extracts. Ungerminated barley (Spratt Archer or Plumage Archer) was ground to a flour and 1000 g. of flour soaked for 2 hr. in 2 l. of ether with occasional stirring. The ether was removed by filtration and the residue washed three times with ether and the excess allowed to evaporate in air. Since aqueous extracts of the flour may have a pH as low as 5.0, at which level phosphorylase may be progressively inactivated, the defatted flour was stirred with 2 l. of 0.02N- Na_2CO_3 and about 50 ml. of toluene. The pH of the mixture fell at first rapidly from about 8 towards 6. A further 200 ml. of 0.2N- Na_2CO_3 was added in lots of 50 ml., as required to maintain the pH between 6.4 and 6.8. After 5-6 hr. the pH was finally adjusted to 6.8, and the mixture allowed to stand in the cold for a further 20 hr. It was then centrifuged at 2200 rev./min. for 40 min. yielding 1250 ml. of orange-coloured slightly cloudy supernatant liquid. In the first experiment, illustrated in Fig. 1, an aqueous extract of grain was employed, in which the pH was adjusted to 6.2 after centrifugation; for all other experiments the procedure designed to minimize phosphorylase losses due to a rather acid pH during extraction was employed.

Measurement of enzyme activities

With one exception (see Fig. 1) experiments were carried out at 30° and in 0.05M-citrate buffer, pH 6.8. Colorimetric measurements were made using an Evans Electro-selenium (EEL) photoelectric colorimeter, with a red filter (Ilford 608, transmission 650-700 m μ).

Phosphorylase activity was estimated by the amount of inorganic phosphate liberated from glucose-1-phosphate. The digests, in addition to buffer, contained 0.038M-glucose-1-phosphate, 0.2% (w/v) soluble starch (or other primer) and amounts of enzyme from 0.1 to 0.5 ml., in a total volume of 1 ml. Samples (0.1 ml.) were withdrawn at suitable time intervals and delivered into 0.9 ml. of 6% (w/v) trichloroacetic acid; inorganic phosphate was determined in filtrates from this mixture by the method of Fiske & Subarow (1925). Results are expressed in terms of percentage of glucose-1-phosphate converted.

β -Amylase activity was estimated by the change in absorption of the blue iodine complex of amylose (McCready & Hassid, 1943). The digests contained, in addition to the buffer, 1 mg. amylose and 0.1 or 0.2 ml. of enzyme in a total volume of 1.6 ml. Samples (0.3 ml.) were withdrawn at suitable time intervals and delivered into about 9 ml. of water containing 0.2 ml. of 0.2% (w/v) I_2 in 2% (w/v) KI and a drop of N-HCl. The volume was made up to 10 ml. before determining the absorption value of the blue solution. One unit of β -amylase is defined as the amount of enzyme required to convert 1 mg. of amylose to maltose in 5 min. at pH 6.8 and 30°.

RESULTS

Action of barley extracts on glucose-1-phosphate

In a series of exploratory experiments, the action of aqueous extracts of ungerminated barley and of sprouting barley on glucose-1-phosphate was examined. An example of the results obtained with ungerminated grain is presented in Fig. 1. The experiments were carried out using maleate buffer at pH 6.0 and at 25°, and the digests were covered

with a layer of toluene. After a lag period of 4-5 hr. there set in a slow conversion of the ester phosphate to another form of organic phosphate which is not susceptible to hydrolysis in 7 min. by N-acid, with simultaneous formation of some inorganic phosphate. At no stage in the process was iodine-staining material detected. The major conversion product has not yet been finally characterized, but evidence to be presented elsewhere suggests that it may be glucose-6-phosphate. Formation of appreciable

Fig. 1. Conversion of glucose-1-phosphate to an ester resistant to hydrolysis by N-acid in 7 min. and release of free phosphate by an aqueous extract of ungerminated barley. Digests at 25°: 20 ml. containing 0.031M-glucose-1-phosphate, 0.05M-maleate buffer of pH 6.0, and 10 ml. barley extract.

quantities of hexose diphosphate appears to be excluded by the virtual absence of easily hydrolysable phosphate in the final stages of the reaction. However this may be, it is clear that aqueous extracts used under these conditions afford no evidence of the presence or absence of phosphorylase.

Action of cereal extracts and of crystalline β -amylase on the activity of broad-bean phosphorylase

The effect of inclusion of 0.25 ml. of cereal extracts, prepared as described for barley, in 1 ml. digests containing bean phosphorylase is illustrated in Fig. 2. Extracts of wheat, oats and rye reduced to about a third or less the conversion of glucose-1-phosphate. With barley extract there was no measurable production of inorganic phosphate at all. This experiment indicates that the cereal ex-

tracts exert an inhibitory action upon phosphorylase and that the failure to produce iodine-staining materials is not simply due to rapid degradation of the synthesized polysaccharide by β -amylase.

The inhibitory action was lost when the cereal extracts were heated to 80° , and this fact together with a further observation that β -amylase preparations from the soya bean (made by Mr S. J. Pirt) were also inhibitory suggested that β -amylase was itself the inhibitor. Accordingly, the effects of addition of varying amounts of barley extract and of crystalline β -amylase upon the activity of bean phosphorylase were compared. The results are presented in Fig. 3. It was found that the extracts from 100 mg. of barley flour, contributing about 4 units of β -amylase, completely prevented release of

barley extracts will tend to make the estimates of β -amylase activity too high, so that precise comparison of the degree of inhibition produced by the crystalline enzyme and the barley extract is not yet possible, but in view of the obvious similarity in the type of effect produced upon phosphorylase activity by these two sources of β -amylase it is concluded that β -amylase is likely to be the agent responsible for inhibition in the cereals. Although only traces of phosphate may be released in the first 2 or 3 hr. of incubation when β -amylase is present; nevertheless, liberation of phosphate continues slowly for prolonged periods of time, up to 48 hr. or more, and eventually, unless the concentration of β -amylase is high, the equilibrium value for the phosphorylase reaction may be approached (see Fig. 3).

Fig. 2. Inhibition of broad-bean phosphorylase by aqueous extracts of cereals. Digests at 30° : 1 ml. containing 0.1 ml. phosphorylase, 0.038 M-glucose-1-phosphate, 0.05 M-citrate of pH 6.8, 2 mg. soluble starch and 0.25 ml. cereal extracts as marked on curves.

measurable amounts of phosphate from glucose-1-phosphate in the time taken for the uninhibited reaction to reach equilibrium. When smaller amounts of amylase were added a series of curves was obtained showing increasing amounts of phosphate release; for example, when about 0.2 unit of β -amylase was present, the degree of inhibition was reduced to 50–60%. Potato phosphorylase behaved in the same way as the bean preparation.

Addition of crystalline β -amylase resulted in the same type of inhibition of phosphorylase activity as that found with barley extract, as evidenced by the curves of Fig. 3. The amounts of β -amylase added formed an inverse sequence with the amount of phosphate released, with one anomalous result where 0.08 unit was added as the crystalline enzyme. The presence of small amounts of α -amylase in the

Fig. 3. Inhibition of broad-bean phosphorylase by crystalline β -amylase and by barley extracts. Digests at 30° : 1 ml. as in Fig. 2, but with varying amounts of crystalline β -amylase or barley extracts added. Approximate number of β -amylase units added marked on curves. $\circ$ — $\circ$, crystalline β -amylase added; $\times$ — $\times$, barley extract added.

Effect of barley extract on the ability of soluble starch and of amylose glycollate pretreated with β -amylase to prime the phosphorylase reaction

Swanson & Cori (1948) have shown that β -amylolysis of both amylose and amylopectin leads to a progressive fall in their priming capacity for the phosphorylase reaction. Examination of its effect upon the soluble starch primer under the conditions of the present experiments was made by exposing the primer to the action of barley extracts for varying times, inactivating the β -amylase by heat and then testing the residual priming capacity of the mixture. A 0.2% (w/v) solution of soluble starch retained 60% of its priming capacity after 24 hr. β -amylolysis, so that if added phosphorylase were in

Fig. 4. Priming capacity for potato phosphorylase II of amylose glycollate pretreated with soya-bean β -amylase. Digests at 30°: 1 ml. containing 0.038 M-glucose-1-phosphate, 0.05 M-citrate at pH 6.8, 0.1 ml. phosphorylase and primers as marked on curves.

Fig. 5. Priming capacity for potato phosphorylase II of soluble starch and amylose glycollate pretreated with β -amylase, after exposure to the action of barley β -amylase. 2 mg. soluble starch or 12.5 mg. glycollate exposed to the action of 0.1 ml. barley extract (approx. 0.4 unit β -amylase) in 0.05 M-citrate at pH 6.8, in the presence of glucose-1-phosphate for 44 hr. β -Amylase then inactivated by heat and phosphorylase added. Digests at 30°: 1 ml. containing 0.038 M-glucose-1-phosphate, 0.05 M-citrate at pH 6.8, 0.1 ml. heated barley extract, 0.1 ml. phosphorylase and primers as detailed below. Curve A, control, soluble starch 2 mg. not degraded by β -amylase; curve B, 12.5 mg. glycollate after 44 hr. exposure to β -amylase; curve C, 2 mg. soluble starch after 44 hr. exposure to β -amylase; curve D, no added primer, heated barley extract present; curve E, no added primer, no barley extract added.

fact operative in systems containing active β -amylase (as in Fig. 3), much greater amounts of phosphate should have been released than those actually found. Primer destruction does not therefore appear to be the principal mechanism of the inhibition.

Fig. 6. Inhibition by barley extract of potato phosphorylase I primed with soluble starch or amylose glycollate pretreated with β -amylase. Digests at 30°: 1 ml. containing 0.038 M-glucose-1-phosphate, 0.05 M-citrate at pH 6.8, 0.1 ml. phosphorylase, and either 2 mg. soluble starch or 12.5 mg. glycollate, in presence or absence of 0.1 ml. barley extract (approx. 0.4 unit β -amylase) as marked on curves.

The discovery of a primer resistant to β -amylolysis has enabled this view to be further tested. Amylose glycollate, pretreated with β -amylase from the soya bean, was found to prime the phosphorylase reaction, but less efficiently than starch. Its efficacy as a primer for potato phosphorylase II is shown in Fig. 4. Since small amounts of α -amylase were present in all digests, and the glycollate is susceptible to this enzyme, it is not possible to state unequivocally what the true priming capacity is, and further it was necessary to determine whether or not α -amylase action had significant effects under the experimental conditions used to demonstrate the inhibition. The effect of exposure to barley extract for 44 hr. of 1.25% (w/v) glycollate and 0.2% (w/v) soluble starch (amounts initially similar in priming capacity) on the activation of potato phosphorylase II is shown in Fig. 5. The digests were heated after 44 hr. to inactivate the β -amylase and phosphorylase then added. The priming capacity of the

glycollate was unimpaired, so that α -amylase action was negligible, but the residual priming capacity of the starch was very low, indicating that in this prolonged time β -amylolysis was nearly complete. When digests containing the two primers were incubated with potato phosphorylase I and active β -amylase, there was in both cases a high degree of inhibition, so that it becomes clear that β -amylase will inhibit the phosphorylase reaction whether or no the primer

A crude extract of ungerminated barley (1250 ml.), prepared as already described (p. 477) by extraction with dilute Na_2CO_3 , was concentrated to 300 ml. by collecting the fraction coming down between 28 and 80% saturation with $(\text{NH}_4)_2\text{SO}_4$ at pH 7.0 and then dissolving this precipitate in 0.25 M-citrate at pH 6.8. Complete separation of the precipitate required standing in the cold for several hours, during which time the pH tended to fall, and it became necessary to readjust it to 6.8 with 0.1 N-NaOH. About half the β -amylase was removed with the material precipitating

Fig. 7. β -Amylase activity and apparent phosphorylase activity of preparations obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation of a concentrate of ungerminated barley extract (see p. 480). (a) First fractionation of concentrate; (b) refractionation of precipitate obtained between 40 and 50% saturation in (a). Activities are those of precipitates obtained between degrees of $(\text{NH}_4)_2\text{SO}_4$ saturation marked on abscissa. β -Amylase units and digests as on p. 477, with 0.1 ml. barley preparations as enzyme. Apparent phosphorylase activity measured as glucose-1-phosphate converted in 30 min. under conditions set out on p. 477, with 0.2 ml. barley preparations as enzyme. O—O, β -amylase units; x---x, percentage glucose-1-phosphate converted.

is susceptible to β -amylolysis, and further that the degree of inhibition does not greatly depend upon the nature of the primer.

Detection of phosphorylase in barley extract

It is apparent from the foregoing experiments that if phosphorylase is present in barley extracts it can only be detected by lowering the ratio of β -amylase to phosphorylase to a point where the inhibition is only partial. In the potato it has been shown (Hanes, 1940b) that most of the α -amylase present can be separated from the phosphorylase by fractionation with ammonium sulphate; accordingly, this procedure was adopted in a first attempt to reduce the amounts of β -amylase in barley extracts.

below 28% saturation, but the concentrated extract still had a β -amylase activity corresponding to 6 units/ml., and under the standard conditions used (p. 477) failed to liberate phosphate from glucose-1-phosphate for periods up to 24 hr.

The concentrate was subjected to two fractionations with $(\text{NH}_4)_2\text{SO}_4$. In the first the material was divided into six portions, namely, precipitates separating at 30% saturation and then at each 10% increase up to 80%. The second fractionation divided each of the first four of these fractions into three to five more, covering degrees of saturation again up to 80%. All operations were conducted as far as possible at about 4°, and with each fractionation the concentration was increased tenfold. The precipitates were dissolved in 0.25 M-citrate at pH 6.8, the final fractions each in a volume of 2 ml.

The β -amylase and apparent phosphorylase activities of the solutions derived from the first fractiona-

tion are recorded in Fig. 7a. In Fig. 7b are shown the activities after the second fractionation of one of these, namely, the precipitate separating between 40 and 50 % saturation. There were rather large losses of total β -amylase activity during fractionation, possibly due to undesirably large rises of temperature during centrifugation. The remaining β -amylase activity was highest in the fractions precipitating at the lowest ammonium sulphate concentrations and fell consistently as this concentration increased (Fig. 7), but no sharp separation of the enzyme was achieved. However, in the solutions derived from the first fractionation a feeble capacity to liberate phosphate from glucose-1-phosphate was observed (Fig. 7a), and this capacity was greatly increased after the second fractionation (Fig. 7b). In both cases the amount of phosphate released was highest in the fraction precipitating between 40 and 50 % saturation, at which concentration the known phosphorylases are precipitated.

Three further tests were made to confirm that phosphorylase was responsible for the liberation of phosphate from glucose-1-phosphate.

First, the reaction was shown to respond to the addition of soluble starch primer as shown in Table 1.

Table 1. *Increase of phosphate production from glucose-1-phosphate on addition of soluble starch to barley extracts fractionated with ammonium sulphate*

(Fractions obtained in second fractionation (Fig. 7b) precipitating between (1) 30 and 40, (2) 40 and 50 and (3) 50 and 60 % saturation. Digests: 1 ml. containing 0.038 M-glucose-1-phosphate, 0.2 ml. barley fractions, with and without 0.2 % (w/v) soluble starch, pH 6.8, temp. 30°.)

Time (min.)	Glucose-1-phosphate converted (%)					
	1		2		3	
	No starch	Starch	No starch	Starch	No starch	Starch
30	2	5	2	21	1	4
45	4	7	4	30	2	6
180	12	15	10	41	5	10

Secondly, the formation of glucose-1-phosphate from starch and phosphate was demonstrated in 4 ml. digests containing 0.061 M-soluble starch, 0.062 M-phosphate, pH 6.6, and 0.5 ml. of the most active barley preparation (derived from the precipitate at 40–50 % $(\text{NH}_4)_2\text{SO}_4$ saturation of the second fractionation). After 2 hr. at 30°, 6 ml. of 10 % (w/v) trichloroacetic acid was added and the precipitate removed as quickly as possible. The solution was at once neutralized with NaOH and free phosphate precipitated with magnesia mixture. Samples of the solution were tested for free phosphate after filtration, in case precipitation was incomplete, and further samples hydrolysed with N -acid for 7 min. and the increase in free phosphate determined. In this way it was found that about 20 % of the added phosphate had been converted to glucose-1-phosphate. No great accuracy is claimed for this figure, as considerable difficulty was experienced in obtaining perfectly clear solutions for the colorimeter readings, but there was no doubt that ester phosphate had resulted from the action of barley extract on starch.

Finally, production of polysaccharide in digests containing 0.27 % (w/v) of achroic primer, 0.038 M-glucose-1-phosphate at pH 6.8, and 0.3 ml. of the barley preparation was indicated by I_2 staining. Drops from 1 ml. digests were tested at frequent intervals; after 5 min. a green-blue colour was found, and after 10 min. and for periods up to about 1 hr. a pure blue. The intensity of colour was not great, and after incubation for more than 1 hr. no colour was given, presumably owing to degradation of the polysaccharide by β -amylase.

DISCUSSION

The detection of phosphorylase in barley extracts adds another of the major starch-storing organs of plants to the number already known in which a phosphorylating mechanism plays a part in starch synthesis. The search for phosphorylase in extracts of ungerminated cereals has revealed difficulties not encountered in investigations of peas and potatoes (Hanes, 1940b; Green & Stumpf, 1942) owing to the fact that in crude aqueous extracts of cereal grains the phosphorylase activity may be completely masked. Two factors contribute to this effect, the rather low pH of the extracts and the presence of large amounts of β -amylase. The low pH leads to instability of the phosphorylase during extraction and it is therefore necessary to extract with suitable

buffers rather than water. The effects of β -amylase on the reaction appear to be threefold. The product of synthesis from glucose-1-phosphate is degraded, and there is also some destruction of the carbohydrate primer necessary to initiate this synthesis, which tends to reduce the rate of polysaccharide production. Finally, an inhibitory action is exerted upon phosphorylase activity independent of the two effects upon carbohydrate. It is of some interest that hitherto the only cereal in which phosphorylase activity has been reported is waxy maize (Bliss & Naylor, 1946), a grain which exhibits little or no β -amylase activity (Kneen, 1944).

The mechanism of this inhibition is not at present clear, but the data of Fig. 3 suggest that the general effect of β -amylase addition is similar to that produced when phosphorylase action proceeds in the presence of suboptimal amounts of primer, a situa-

tion which results in the production of relatively long polysaccharide chains by attachment of glucose-1-phosphate residues to a relatively small number of primer end groups (Cori, Swanson & Cori, 1945). The view that available end groups are reduced by the presence of β -amylase is supported by the change in type of polysaccharide obtained when β -amylase of barley is added to potato phosphorylase. Thus in a digest containing 0.038M-glucose-1-phosphate and primed with a mixture of achroic dextrans the action of potato phosphorylase I yielded at equilibrium a polysaccharide which stained red-purple with iodine, and only further additions of ester lengthened the chains sufficiently to produce a blue-staining polysaccharide. When barley β -amylase was added to a similar digest, in amounts which were only partially inhibitory, the polysaccharide formed gave with iodine a weak but bright blue colour, indicating that only a few relatively long chains were formed.

Effective reduction of end groups for synthesis may presumably be brought about either by interaction of the β -amylase with the primer itself or with the active centres of the phosphorylase. In the latter case the possibility presents itself that, in the absence of phosphate, phosphorylase in its turn might influence the course of β -amylolysis. An attempt to test this point was made by adding phosphorylase to digests containing amylose and crystalline β -amylase. Unfortunately, this procedure revealed the fact that none of the available phosphorylase preparations was completely free from α -amylase, and the accelerating effect on β -amylolysis of even minute traces of this enzyme was sufficient to account for the acceleration of amylose breakdown under the conditions tried. Since this accelerating effect is well known it has not been considered necessary to record the data collected in these trials.

The account presented here can only be regarded as a preliminary report of the existence of the in-

hibitory mechanism, but the observations would appear to afford a good example of what may well be a general problem in dealing with plant extracts, namely, that failure to demonstrate activity in a crude extract cannot be regarded as an indication of the absence of the enzyme sought.

SUMMARY

1. Aqueous extracts of ungerminated barley convert glucose-1-phosphate into an ester resistant to hydrolysis by N-acid in 7 min. with the simultaneous release of some inorganic phosphate. No iodine-staining polysaccharides are produced.

2. Extracts of wheat, oats, rye and barley grains inhibit the activity of broad-bean and potato phosphorylase. The inhibitory power is related to the amount of β -amylase present, and is destroyed by heating the extracts. Soya-bean β -amylase and crystalline β -amylase from the sweet potato (Balls) have the same inhibitory effect.

3. The inhibition is not due to destruction of primer by β -amylase.

4. Phosphorylase can be detected in extracts of ungerminated barley made at pH 6.8, after fractionation and concentration with ammonium sulphate. This procedure reduces the ratio of β -amylase to phosphorylase to a level where inhibition is only partial.

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