EFFECT OF DIFFERENT POLYSACCHARIDES AND POLYSACCHARIDE DEGRADATION PRODUCTS ON THE ACTIVITY OF POTATO PHOSPHORYLASE*

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A synthetic polysaccharide has been obtained from glucose-1-phosphate by the action of a phosphorylase from muscle (1) or one from potatoes (2, 3). Both enzymes appear to produce the same polysaccharide (4). The polysaccharide has been termed a synthetic “starch,” because a similar, if not identical, product can be obtained by fractionation of natural starches by means of butanol, isoamyl alcohol, or other polar solvents (5). This fraction, which is generally called amylose (6), appears to be made up of long unbranched chains of glucopyranose units in 1,4-α-glucosidic linkages (4).

Studies on the enzymatic synthesis showed that it was necessary to activate the phosphorylases by having present small amounts of soluble starch or glycogen (1-3). Obviously, it is important to understand the rôle of such activator carbohydrates in polysaccharide synthesis. Synthetic amylose polysaccharides have been regarded as incapable of activating either potato (2, 3) or muscle (4, 7) phosphorylase. Various natural starches, glycogen, and some dextrins have activating ability for the potato enzyme (3, 8). The Cori (7) group believes that, “Polysaccharide synthesis [by muscle phosphorylase] might consist in a lengthening of existing side chains [of glycogen or natural starch] by addition of glucose units in 1:4 glucosidic linkages.” The apparent inability of amyloses to act as activators is attributed to the low concentration of end-groups and low solubility. Since the polysaccharides formed by the enzymes of potatoes and muscle are so similar, it might be assumed that the potato phosphorylase also requires branched chain activators of the amyllopectin type.

The purpose of the present investigation was to determine the effect of partial acid hydrolysis, and certain other factors, on the ability of

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different polysaccharides to activate potato phosphorylase. Preliminary
to this, however, it seemed advisable to investigate the preparation of
suitable enzyme concentrates and the determination of enzyme activity.
Therefore, the results of such studies are also given here. Some of the
data obtained in this investigation have been reported in a preliminary
note (9).

EXPERIMENTAL

Materials—All the glucose-1-phosphate was prepared by the method
of Hanes (2) and recrystallized until the [α]$_{20}^{20}$ of the dipotassium salt di-
hydrate was 75–79°.

Potato phosphorylase was purified by fractional precipitation with
ammonium sulfate. A sufficient quantity of potatoes (Solanum tuberosum)
was peeled and sliced to furnish at least 1 liter of juice. They were then
kept under water until used, usually within 1 hour. Then they were
drained quickly and pulped in a Waring blender. The crude juice was
brought to a specific gravity of 1.085 with solid ammonium sulfate and
centrifuged within $\frac{1}{2}$ hour. The supernatant liquid was raised to a
specific gravity of 1.152 with ammonium sulfate and then recentrifuged.
The precipitate, which was not entirely soluble in water, was suspended
in 250 ml. of water and fractionation was repeated in a specific gravity
range of 1.095 to 1.145. The precipitated enzyme was dissolved in 100
ml. of water for two subsequent fractionations in the specific gravity
ranges of 1.100 to 1.140 and 1.100 to 1.135 respectively. The pH was
determined at frequent intervals throughout the preparation and was
kept between 6.0 and 6.5 by the addition of dilute ammonium hydroxide.
Inactivation of the enzyme is rapid below pH 5.8.

To preserve the final enzyme precipitate for subsequent use it was
generally taken up in water and diluted to 25 ml. for each liter of crude
potato juice used. The dark solution was stored at 1–2°. No suitable
preservative was found. Merthiolate (Lilly) and thymol caused marked
inactivation. Owing to the occasional need for maintenance of the enzyme
at water bath temperatures for considerable periods of time, the stability
of the most concentrated preparations was studied at 38°. At pH 6.2
no appreciable decrease in activity occurred for 32 minutes. The activity
scarcely changes for 4 to 6 weeks when stored at 1–2°.

Experience has shown that one of the most important precautions in
the concentration of the enzyme is a speedy separation of the freshly
expressed juice from the pulp. Inactivation is particularly rapid at this
stage. All other steps in the procedure should be carried out as fast as
practicable.

An attempt to prepare colorless concentrates was made with $p$-amino-
benzoic acid and sulfanilamide to inhibit tyrosinase activity in the juice (10). Although \( p \)-aminobenzoic acid was partially effective, it was not regarded as satisfactory enough to warrant its use in this work. Sulfanilamide had no effect.

Nitrogen was determined on aliquots of the various fractions by the micro-Kjeldahl method. Protein was precipitated in a 15 ml. Pyrex centrifuge tube with trichloroacetic acid and washed three times, by centrifugation, to remove ammonium sulfate. Acid digestion was carried out in the tube. The purest preparations showed an activity of 18.2 units per mg. of nitrogen. This appears to be almost identical with the activity of Green and Stumpf’s (3) most active preparations.

Repeated preparations of the enzyme concentrate by this means have demonstrated good reproducibility of results, an accomplishment difficult to achieve when the quantity of ammonium sulfate to be added in each fractionation is determined by the degree of saturation. Therefore, this method of concentration seems to be more satisfactory than the customary procedure (3) which is based on the addition of ammonium sulfate to specified degrees of saturation.

Synthetic polysaccharide was prepared by incubating about 280 units (as determined by our method) of purified potato phosphorylase in 0.25 M citrate buffer at pH 6.2 with 12.5 gm. of dipotassium glucose-1-phosphate dihydrate at 37°. The total volume of the reaction mixture was about 250 ml. The insoluble polysaccharide that formed was centrifuged off after about 24 hours, washed several times with water and then with alcohol, and dried at 70°. The yield was 3.2 gm. or 62.5 per cent of the quantity theoretically possible at that pH. The nitrogen content could not have exceeded 0.48 per cent, because the added phosphorylase contained only 15.4 mg. of nitrogen.

The linear fraction of corn-starch (amylose) was prepared according to the method of Schoch (5).

Modification of Green and Stumpf’s Method for Determination of Phosphorylase Activity—According to Green and Stumpf (3), the activity of potato phosphorylase is directly proportional to the concentration of the enzyme even when the activity is great enough to liberate approximately 1 mg. of inorganic phosphorus per 3 minutes. When the reaction is allowed to run 5 to 10 minutes, as recommended for their method, the total amount of phosphorus liberated will be, by calculation, about 1.7 to 3.3 mg., if the reaction is strictly linear under such conditions. Since the total amount of phosphorus furnished by the glucose-1-phosphate used in their determinations is only 3.1 mg., and at equilibrium not more than 80 per cent can be inorganic, it is obvious that the permissible enzyme concentration must be more precisely defined.
Figs. 1 to 4 show the effects of enzyme concentration, temperature, added polysaccharide, and glucose-1-phosphate concentration on the rate of phosphate liberation. These data demonstrate that the activity is not directly proportional to the enzyme concentration when the latter is great enough to cause the liberation of much more than 1.0 mg. of phosphorus.

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1 Data necessary for the preparation of Fig. 4 were obtained by Miss Elsa Proehl.
phorus per 10 minutes (Fig. 1). Also, it is worthy of note that the temperature coefficient is approximately 2.0 at the temperature interval 10-20° and it approaches 1.3 at 40-50° (Fig. 2). Thus the enzyme behaves like many others in this respect (11). Green and Stumpf's (3) finding that 10 to 20 mg. of soluble starch provide for maximal activation of the enzyme was confirmed (Fig. 3). As shown in Fig. 5, there is no autocatalytic action in the synthesis of polysaccharide from glucose-1-phosphate by potato phosphorylase.

On the basis of these data we have used as the unit of phosphorylase activity that amount of enzyme which will liberate 0.1 mg. of inorganic phosphorus in 3 minutes from glucose-1-phosphate at 38° and pH 6.2. The reaction mixture is composed of 1.0 ml. of enzyme solution of such dilution that not more than 0.5 mg. of phosphorus will be liberated in 10 minutes, 0.5 ml. of 1.0 M citrate buffer of pH 6.2, 1.0 ml. of 2 per cent soluble starch solution; 1.0 ml. of 0.1 M glucose-1-phosphate is added to the rest of the mixture after both have been brought to temperature equilibrium. The period of incubation is 10 minutes. The reaction is halted by rapid addition of 2.5 ml. of 10 per cent trichloroacetic acid, and phosphorus is determined on an aliquot of the reaction mixture by the Fiske and Subbarow method (12).

Extraction of the reactants with diphenylthiocarbazone (dithizone) in carbon tetrachloride at pH 6.2 had no effect on the activity of the enzyme. Also, manganous chloride, cobaltous sulfate, nickelous sulfate, thiamine, urea, p-aminobenzoic acid, and sulfanilamide, in moderate concentrations, were without effect. Thus it appears that the concentration of various heavy metals and certain common organic substances is not of importance in phosphorylase determinations, unless extraordinary quantities are present.
ACTIVITY OF POTATO PHOSPHORYLASE

Effect of Acid Hydrolysis on Ability of Different Polysaccharides to Activate Phosphorylase—It was reasoned that the reported inability of synthetic polysaccharide to act as an activator might be due to structural arrangements causing an inadequate concentration of groupings necessary for that function. It seemed likely that partial acid hydrolysis of the synthetic material, or dispersion with alkali, might produce active substances.

A typical experiment with acid is described. After correcting for ash, 2.5 gm. of starch were suspended in 18 ml. of water in a 50 ml. volumetric flask. The flask and contents were chilled and then concentrated hydrochloric acid, also chilled, was added to the mark. The mixture was agitated continuously until all the polysaccharide was in solution. This required 3 to 4 minutes. The temperature was kept at 27°. At the end of 5 minutes the first sample, 1.0 ml., was removed and transferred to a 10 ml. volumetric flask in which had been placed nearly enough 1.65 N sodium hydroxide to neutralize the acid. By adding small amounts of sodium hydroxide the neutralization was quickly completed and water was added to the mark. Constancy of pH in subsequent neutralized samples was assured by transferring 1.0 ml. aliquots (equivalent to 5 mg. of starch) to test-tubes, each containing 0.5 ml. of the citrate buffer at pH 6.2. The activating power was determined as described earlier, with the neutralized samples in place of soluble starch in the test mixtures.

As indicated in Figs. 6 and 7 the activating ability of the hydrolyzing polysaccharide solutions increased progressively to a maximum and then gradually declined. The reducing value (copper) of the solutions became maximal after all activating ability had been lost. The iodine-staining reaction of the hydrolysates disappeared by the 4th to 9th hour. The achromic point was reached quickest by hydrolyzing corn-starch and slowest by the linear component of corn starch (amylose). The activating ability began to decline almost simultaneously with disappearance of the iodine color reaction. Potato and arrowroot starches, when similarly treated, gave almost identical results to corn-starch (Fig. 6).

Higher concentrations of acid caused the maximal activating effect to be reached sooner, and, in other respects also, the effects on the polysaccharides were telescoped. This is shown by Fig. 7 which summarizes an experiment in which the normality of the acid solution was increased to 10.1. All other conditions were the same as previously described. Under such conditions the achromic point was reached in less than 2 hours and the activating ability passed from a high value to almost zero in less than 5 hours.

In some other experiments the partially hydrolyzed activators were tested in concentrations of 0.5 mg. per determination. The principal results have been reported briefly (9). They show that each substance
attains approximately the same level of activating power, but it requires a shorter period of hydrolysis for the corn-starch to reach maximal activity than the essentially pure amylases, "synthetic" potato starch, and Schoch's butanol-insoluble fraction of corn-starch.

The data indicate that activation of potato phosphorylase is not dependent upon branched chain polysaccharides, because the partial hydrolysis of synthetic polysaccharide and the butanol-insoluble fraction of corn-starch yields activators as effective as similarly treated natural starches that are rich in branched chain glucose units. Also, the results with achroodextrins suggest that the carbohydrate activator of potato phosphorylase need not contain more than 7 or 8 glucose residues per molecule, because Hanes and Cattle (13) have reported that polysaccharides may be fragmented to dextrins which appear to contain only 7 to 8 glucose units before giving an achromic iodine test. Thus it seems probable that purer synthetic amylose could be obtained by means of partially acid-hydrolyzed amylose as the activator of potato phosphorylase. The preparations made by present methods are impure (4).

Although the conditions employed for acid hydrolysis may cause the condensation of some glucose into branched chain structures (14) it does not seem possible that this could account for the enzyme activation, because the latter effect subsides and eventually disappears, whereas the condensation products remain.
Effect of Alkali on Activating Ability of Synthetic Potato Polysaccharide—
To 50 mg. of the synthetic material suspended in about 5 ml. of water 1 drop of concentrated potassium hydroxide was added. The polysaccharide dissolved almost immediately. The solution was quickly neutralized with hydrochloric acid and diluted to 10.0 ml. The activating ability was found to be within the range of corn-starch hydrolyzed with hydrochloric acid for 10 minutes (Table I). After 48 hours extensive retrogradation had occurred and the supernatant solution was scarcely more active than a saturated aqueous solution of untreated synthetic polysaccharide.

Degree of Acid Hydrolysis of Activating Polysaccharide and Iodine Color Reaction of Synthesized Products—The unexpected results of a lecture demonstration led us to consider the effect of the activator polysaccharide on the nature of the synthesized product as indicated by the reaction of the latter with iodine. Sumner et al. (8), in a preliminary note, reported that "the nature of the product synthesized from Cori ester by plant phosphorylase depends upon the kind and amount of carbohydrate added to prime the reaction."

Two sources of activator polysaccharides were used, the non-dialyzable dextrins from fat-free corn-starch which had been hydrolyzed to the achromic point with hydrochloric acid, and synthetic polysaccharide hydrolyzed in stages ranging from 5 to 750 minutes. The dextrins were prepared by neutralizing the hydrolyzed starch with sodium hydroxide, dialysis until free from chlorides, concentration in vacuo to a small volume, and then precipitation with methanol. The precipitate was dried and used in the concentrations given in Table II. To determine the activating ability of the dextrins and partially hydrolyzed synthetic polysaccharide, the same methods were used as described previously. At the end of the incubation periods, ranging from 5 to 40 minutes, the reactions were stopped by adding 0.2 ml. of 0.5 per cent iodine in 1.0 per cent potassium iodide. The absorption characteristics of the different samples were studied by means of a Coleman spectrophotometer.
As indicated in Table II the color formed by iodine and the carbohydrate synthesized during the first few minutes tended to be about the same as that given by iodine and the activator polysaccharide, even though the latter ranged from blue to colorless, depending upon the degree of acid hydrolysis it was subjected to. However, prolongation of the polysaccharide-synthesizing reaction results in products that stain blue with iodine irrespective of the character of the activator. One possible explanation seems to be that the synthesis is essentially the formation of amylose type chains in combination with the activator polysaccharide, somewhat as suggested by Cori \textit{et al.} (7), and that, within limits, the length of the chain is determined by the reaction time. Thus a short reaction time, especially with large amounts of activator, would yield amylodextrins giving a different color with iodine than products formed by prolonged enzymatic action.

**Table II**

\textbf{Degree of Acid Hydrolysis of Activating Polysaccharide and Iodine Color Reaction of Synthesized Products}

<table>
<thead>
<tr>
<th>Activator</th>
<th>Activity (inorganic P liberated in 10 min.)</th>
<th>Iodine color reaction of Synthesized products formed during incubation periods of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min.</td>
<td>mg.</td>
</tr>
<tr>
<td>Synthesized potato polysaccharide</td>
<td>20</td>
<td>0.037</td>
</tr>
<tr>
<td>hydrolyzed for</td>
<td>510</td>
<td>0.405</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>0.098</td>
</tr>
<tr>
<td>Dialyzed corn-starch dextrin</td>
<td>0.5</td>
<td>0.224</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>0.629</td>
</tr>
</tbody>
</table>

**SUMMARY**

A convenient method of concentrating phosphorylase from potatoes has been described. The factors of importance in determining phosphorylase activity have been studied and on the basis of the results slight modifications of the Green and Stumpf analytical method have been made.

The ability of different polysaccharides to activate potato phosphorylase increases progressively to the neighborhood of the aëromic point, when hydrolyzed with acid, and then diminishes as hydrolysis is continued.
Amylose type polysaccharides, both natural and synthetic, exhibit this behavior as well as starches containing both branched and straight chain components. It appears probable that the phosphorylase activator need not contain more than 7 or 8 glucose units per molecule. Branched chain polysaccharides are unnecessary for the activation of potato phosphorylase.

Some attempt has been made to determine whether activator polysaccharides constitute patterns which regulate the nature (molecular weight, structural configuration, etc.) of the synthesized polysaccharides. The results are inconclusive.

**BIBLIOGRAPHY**

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