Purification and Mechanism of Action of Sucrose Phosphorylase*

(Received for publication, October 21, 1966)

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SUMMARY

Sucrose phosphorylase (disaccharide glucosyltransferase) from Pseudomonas saccharophila has been highly purified. The enzyme appears to be homogeneous in the ultracentrifuge, on starch gel electrophoresis and on polyacrylamide gel disc electrophoresis. The molecular weight of the enzyme as determined by Sephadex chromatography is 80,000 to 100,000.

The kinetics of the reaction was examined and found to be consistent with a double displacement mechanism involving formation of a glucose-enzyme complex and a subsequent reaction of this complex with acceptor to form the reaction products. Further evidence of the formation of a glucose-enzyme complex was obtained by isolating a de-natured form of the complex. This complex contained 1 mole of glucose per mole of enzyme.

Sucrose phosphorylase (disaccharide glucosyltransferase, EC 2.4.1.7) catalyzes a number of glucosyl transfer reactions (1-4), for example, sucrose + P_i = α-D-glucose-1-phosphate + D-fructose. In addition to P_i and D-fructose, L-sorbose, D-xylulose, L-arabinose, L-arabinulose, AsO_3^- or H_2O can also serve as glucosyl acceptors. The reaction with AsO_3^- or H_2O leads to the formation of glucose and is irreversible. It has been shown that sucrose phosphorylase also catalyzes the exchange reaction of P_i with α-D-glucose-1-P (1) and of D-fructose-14C with sucrose (5). The discovery of the P_i exchange reaction led to the postulate that the reactions catalyzed by sucrose phosphorylase involve the intermediate formation of a glucosyl enzyme which then reacts with acceptor to form products. This was the first instance in which isotopic exchange reactions were used to deduce the existence of an enzyme-substrate complex, and in which such a complex was proposed for transfer reactions. Another aspect of this reaction which has been investigated is the site of bond cleavage. This was shown to occur between oxygen and carbon atom 1 of glucose (6).

The exchange experiments, although consistent with the mechanism of Doudoroff, Barker, and Hassid (1), are subject to alternative interpretations. We therefore carried out experiments designed to support or refute the proposed mechanism. These experiments as well as the preparation of highly purified sucrose phosphorylase are reported here. This enzyme preparation has approximately 30 times the activity of a previously described preparation from Pseudomonas saccharophila, and has approximately the same specific activity as the enzyme from Leuconostoc mesenteroides (4).

EXPERIMENTAL PROCEDURE

Analytical Procedures—Sucrose phosphorylase was assayed by measuring the rate of reduction of TPN^+ in a coupled system consisting of sucrose phosphorylase, phosphoglucomutase, and glucose-6-P dehydrogenase. One unit of enzyme activity is defined as the amount of enzyme that will reduce 1 μmole of TPN^+ per min per ml under the assay conditions. The assay mixture was composed of 0.05 mM potassium phosphate (pH 7.0), 0.08 mM sucrose, 6 x 10^-4 M magnesium sulfate, 0.01 mM cysteine (neutral), 5 x 10^-4 M TPN^+, 50 μg per ml of phosphoglucomutase (Sigma), 5 μg per ml of glucose-6-P dehydrogenase (Calbiochem, Grade A), and between 0.0005 and 0.005 unit per ml of sucrose phosphorylase. Total volume was 1 ml. Under these conditions, the rate of reduction of TPN^+ was linear with sucrose phosphorylase concentration. The assay was carried out at 30℃.

α-D-Glucose-1-P was assayed spectrophotometrically by a method similar to that described by Bergmeyer and Klotzsch (7). The sample was incubated in a reaction mixture that contained 0.04 M Tris-chloride buffer (pH 8.0), 10^-3 M MgCl_2, 5 x 10^-4 M TPN^+, 5 μg per ml of glucose-6-P dehydrogenase, and 50 μg per ml of phosphoglucomutase. Glucose was determined with the Glucostat (Worthington) or with glucose-6-P dehydrogenase. For the enzymatic determinations, the reaction mixture contained 0.04 M Tris-chloride buffer (pH 8.0), 10^-3 M MgCl_2, 5 x 10^-4 M TPN^+, 5 μg per ml of glucose-6-P dehydrogenase, 10 μg per ml of hexokinase, 5 x 10^-4 M ATP, and between 0.02 and 0.12 μmole per ml of glucose. In some cases, when no interfering substances were present, glucose was determined by the ferricyanide method (6).

The ferricyanide procedure was also used for the determination of fructose and total glucose plus fructose. Fructose and total glucose plus fructose were also determined enzymatically in the system used for the glucose assay with the addition of 10 μg
per ml of phosphoglucosisomerase. Sucrose was determined with a non-specific carbohydrate assay involving the reaction with phenol and concentrated sulfuric acid (9). Orthophosphate was determined by the method of Lowry and Lopez (10). Under these conditions, α-n-glucose-1-P is not hydrolyzed. Protein assays were carried out by ultraviolet absorption (11) and by the method of Lowry et al. (12).

Kinetic Measurements—All reactions were carried out at 30° in 0.03 M Tris-maleate buffer, pH 7.0. Reactions were generally followed for 10 min; aliquots were removed at intervals and the reaction was quenched by dilution into H₂O heated to about 80° in a sand bath. These aliquots were then assayed for the amount of product formed. Rates were obtained from four to six time points and were always linear for the time period followed. In general, less than 10% of the substrate was utilized during a reaction. In some cases, in which two measurable products were formed, both were assayed and the results were found to be consistent. The reactions were initiated by addition of substrate to the enzyme reaction mixture. Zero time points were obtained by adding substrate to the hot water first and then adding the complete reaction mixture (minus substrate) to the hot solution.

Synthesis of Sucrose-14C Labeled in Fructose Moiety—n-Fructose-14C (uniformly labeled), 200 μC of specific activity 242 μC per μmole (4.6 × 10⁵ cpm per μmole), which was obtained in 90% ethanol (New England Nuclear), was evaporated to dryness with a rotary evaporator. To the dry n-fructose-14C (uniformly labeled) were added 5.1 mg of sucrose in 0.1 ml of water, 1.5 units of sucrose-phosphorylase in 0.1 ml of 0.005 M potassium-nitrate buffer (pH 7.0), 0.04 ml of 0.1 M n-fructose, and 0.06 ml of water. The final volume was 0.3 ml. The above mixture was allowed to react for 1 hour and then was quenched by swirling the reaction flask in boiling water for 1 min. The reaction mixture was then streaked on two water-washed Whatman 3MM, chromatography papers, 9 × 22 inch, in 6-inch streaks. Sucrose and fructose markers were placed on both sides of each streak. The papers were developed in a descending system for 20 hours with isopropanol-water (160:40) as developing solvent (13) and the solvent was allowed to drip off the edges of the papers. The outside marker strips were cut off and stained with silver nitrate (14). The sucrose-14C streaks were thereby found, cut out, eluted with water, and rechromatographed as above. The final chromatogram revealed only one radioactive spot, which cochromatographed with sucrose. The final product was eluted from the papers as described above and was found to have a specific activity of 1.4 × 10⁶ cpm per μmole.

Growth of P. saccharophila (ATCC 9114)—The bacteria were grown according to a modification of the method of Doudoroff (15) in a medium containing 0.033 M KH₂PO₄-Na₂HPO₄ buffer (pH 6.5), 0.1% ammonium chloride, 0.05% magnesium sulfate, 0.005% ferrie ammonium sulfate, 0.001% calcium chloride, and 0.5% sucrose. Growth is initiated with a 10% inoculum. The resulting cell soup was suspended in 50 gallons of original medium and centrifuged again in a De La Val continuous flow centrifuge. The centrifuge was stopped without disturbing the cell paste. This paste was then frozen. The bacteria may be kept in this state for more than 6 months without a significant loss of enzyme activity.

Enzyme Purification.—Bacterial paste, 1,100 g, was suspended in 800 ml of 0.1 M potassium phosphate, pH 7.0. The cells were sonically disrupted in a continuous flow cell for 1.5 hours at a flow rate of 100 ml per min in an MSE 500-watt sonic oscillator. The temperature was maintained between 5° and 20°. After sonication the suspension was centrifuged for 1 hour at 7,000 × g and the cell debris was discarded. To the supernatant fluid (crude sonic extract) was added sufficient 8% protamine sulfate solution (Krishill) to give 0.38 mg of protamine sulfate per mg of protein. The precipitate was removed by centrifuging for 30 min at 20,000 × g and discarded.

The supernatant was brought to 50%, saturation with ammonium sulfate by addition of neutral, saturated (0-5°) ammonium sulfate. The suspension was stirred for 30 min and then centrifuged at 20,000 × g for 30 min. The supernatant was discarded and the precipitate was dissolved in sufficient 0.005 M potassium citrate buffer, pH 5.7, to give a final volume of 200 ml. The solution was dialyzed against three changes of 0.005 M potassium citrate, pH 5.7, each change consisting of 8 liters. Any resulting precipitate was removed by centrifuging at 20,000 × g for 30 min.

The extract was next diluted to 16 mg per ml with 0.005 M potassium citrate buffer, pH 5.7, and applied to a DEAE-cellulose column that had been equilibrated with 0.005 M potassium citrate buffer, pH 5.7. The DEAE-cellulose occupied a volume of 1.7 liters after packing under pressure, and the height to diameter ratio of the column was about 10. The column was washed with 0.005 M potassium citrate buffer, pH 5.7, until the optical density of the effluent at 280 μg was less than 0.1. A convex exponential gradient was then applied to the column. The lower reservoir, containing 0.005 M potassium citrate (pH 5.7), was 4 liters, and the upper reservoir, containing 0.4 M sodium chloride in 0.005 M potassium citrate (pH 5.7), was 6 liters. Under these conditions, the enzyme activity was eluted on the trailing edge of a large protein peak.

After the enzyme pool was brought to pH 7 by the addition of 1 M potassium phosphate (dibasic), the total volume was 750 ml. The enzyme was then precipitated by the addition of 234 g of solid ammonium sulfate. The solution was maintained at pH 7 during the precipitation by the addition of 1 M KH₂PO₄. The suspension was allowed to stand for 1 hour and then centrifuged at 20,000 × g for 30 min. The precipitate was dissolved in sufficient 0.005 M potassium phosphate buffer, pH 6.5, to give a final volume of 30 ml, and was dialyzed against two 2-liter volumes of 0.005 M phosphate buffer, pH 6.5.

Two calcium phosphate columns were prepared according to the method of Massey (16). Whatman ashless cellulose powder, 25 g, and 125 ml of a calcium phosphate gel suspension (17) containing 70 mg of solid per ml were added to 250 ml of 0.005 M potassium phosphate, pH 6.5. The resulting slurry was degassed under vacuum and then poured into a column 4 cm in diameter containing a scintened glass disc and a 1-inch layer of cellulose powder at the bottom, and allowed to stand in the
cold until settled (about 48 hours). Potassium phosphate, 0.005 M, 500 ml (pH 6.5), was then passed through the column to equilibrate it. The flow rate of the column was about 12 ml per hour. The dialyzed, concentrated enzyme solution was divided into two portions, each containing a total of 500 mg; each was applied to a separate column. The columns were washed with 100 ml of 0.005 M potassium phosphate, pH 6.5. The enzyme was then eluted with 0.012 M potassium phosphate, pH 6.5, and the tubes containing the activity peaks were pooled. The enzyme solution was brought to a protein concentration of approximately 10 mg per ml by placing it in dialysis tubing and embedding the bag in cold, dry silicic acid powder. The bag was checked every 2 hours and retied to reduce surface area and to prevent portions from drying out. The solution was then dialyzed against two changes of 0.005 M potassium acetate, pH 5.5. A carboxymethyl cellulose column was prepared such that the diameter of the column was one-tenth of its height and its volume was about 400 ml of packed carboxymethyl cellulose. The column was equilibrated with 0.005 M potassium acetate, pH 5.5. The protein solution was applied and the enzyme was eluted in the same buffer. The tubes containing the enzyme peak were pooled and the solution was neutralized to pH 7.0 by the addition of 1 M Tris base, making the volume 76 ml. The solution was made 0.005 M in Tris-maleate buffer, pH 7.0, by the addition of 4 ml of 0.1 M Tris-maleate, pH 7, and concentrated with silicic acid, as described above, to a total volume not over 1.5 ml. It was then applied to a Sephadex G-200 column (2.2 x 100 cm) which had been equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. Those tubes containing eluent of specific activity higher than 50 were pooled, concentrated, and dialyzed exhaustively against 0.005 M Tris-maleate buffer, pH 7.0. A diagram of a typical purification is shown in Table I.

**Table I**

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Amo/Am</th>
<th>Protein mg/ml</th>
<th>Activity units/ml</th>
<th>Specific activity units/mg</th>
<th>Total activity units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude sonic extract ......</td>
<td>0.56</td>
<td>42.0</td>
<td>8.0</td>
<td>0.19</td>
<td>5200</td>
</tr>
<tr>
<td>2. Protamine precipitate ......</td>
<td>1.00</td>
<td>36.0</td>
<td>6.9</td>
<td>0.19</td>
<td>4400</td>
</tr>
<tr>
<td>3. (NH₄)₂SO₄ precipitate ......</td>
<td>1.35</td>
<td>62.0</td>
<td>19.7</td>
<td>0.36</td>
<td>3940</td>
</tr>
<tr>
<td>4. DEAE-cellulose column ......</td>
<td>1.31</td>
<td>0.77</td>
<td>2.49</td>
<td>3.12</td>
<td>1850</td>
</tr>
<tr>
<td>5. (NH₄)₂SO₄ + dialysis ......</td>
<td>1.50</td>
<td>11.0</td>
<td>35.0</td>
<td>3.15</td>
<td>1660</td>
</tr>
<tr>
<td>6. (Ca)₃(PO₄)₂ gel column ......</td>
<td>1.64</td>
<td>0.17</td>
<td>5.5</td>
<td>33.1</td>
<td>1630</td>
</tr>
<tr>
<td>7. Carboxymethyl cellulose column ......</td>
<td>1.44</td>
<td>0.35</td>
<td>13.7</td>
<td>39.0</td>
<td>1040</td>
</tr>
<tr>
<td>8. Sephadex, concentration and dialysis ......</td>
<td>1.76</td>
<td>5.70</td>
<td>337.0</td>
<td>59.0</td>
<td>400</td>
</tr>
</tbody>
</table>

Thixin was suspended in the solution with an electric blender. Quenching by base and Thixin was determined by counting several known radioactive samples in both regular and basic gel systems, and by adding known amounts of radioactivity to one of a duplicate set of unknowns.

**Determination of Molecular Weight**—Molecular weight was determined according to the method of Ackers (18) with a Sephadex G-200 column, 2.2 X 50 cm, equilibrated with 0.05 M Tris chloride buffer, pH 7.5, containing 0.1 M KCl and operated at a flow rate of 0.5 ml per min. The column was calibrated with Blue Dextran 2000 (Pharmacia), ovalbumin (Sigma), and sucrose. Ovalbumin was estimated by the method of Warburg and Christian (11) and sucrose phosphorylase was assayed enzymatically.

**RESULTS**

**Properties of Purified Sucrose Phosphorylase**—The homogeneity of the enzyme was examined by ultracentrifugation, starch gel electrophoresis, and acrylamide gel disc electrophoresis. The enzyme showed a single peak in the ultracentrifuge (Fig. 1), corresponding to a sedimentation constant of 5.2 S. Starch gel electrophoresis, pH 7.0, for 10 hours according to the method of Fine and Costello (19) revealed only one protein spot on staining with 0.1% Amido black. This spot was shown to correspond to the enzymatic activity by cutting the gel into segments, suspending the segments in 0.1 M potassium phosphate buffer (pH 7.0), and assaying for enzymatic activity. Polyacrylamide gel disc electrophoresis, at pH 6.9 to 8.9, by a slight modification of the method of Davis (20) yielded one band. When the gel was overloaded with respect to this band, three slight impurities could be discerned, which could account for less than 10% of the total protein. By cutting the gel into segments, suspending the segments in 0.1 M potassium phosphate (pH 7.0), and assaying for enzymatic activity, the major band was found to correspond to the enzymatic activity.

The molecular weight was estimated by obtaining the Stokes radius and thus the diffusion coefficient of sucrose phosphorylase by the method of Ackers (18). A Stokes radius of 3.7 X 10⁻⁷ cm ± 3% was obtained. This value, combined with an estimated partial specific volume of 0.74 and a sedimentation coefficient of 5.2, gave a molecular weight of 84,000. In view of the assumptions used in calculating the molecular weight, the value obtained can only be considered approximate.

The pH optima for the phosphorylase and hydrolysis were determined. The results are shown in Fig. 2. The pH optimum for hydrolysis was found to be 6.3, while that for phosphorylase was 7.0. This difference may be due to the fact that in the phosphorylase reaction the nature of the acceptor changes with pH, whereas in the hydrolytic reaction it does not. An alternate interpretation of this difference would be that different rate-limiting steps occur in the two reactions.

**Reaction Kinetics**—The kinetics of several reactions catalyzed by sucrose phosphorylase was examined for consistency with Mechanisms I and II. Both of these mechanisms could account for results that have already been reported for isotope exchange (1, 5).

**Mechanism I**

\[
G-X + E \xrightarrow{k_1} G-X \cdot E \xrightarrow{k_2} \text{Products}
\]
where G-X and G-Y represent glucosyl donors, X and Y represent glucosyl acceptors, and E represents enzyme. These mechanisms contain the minimum number of steps necessary to describe the events that must take place if either the double (I) or single displacement mechanism (II) is operative. To describe the reaction fully, it may eventually be necessary to include additional equilibria. The rate expressions for the above mechanisms have been derived (21). The initial velocity in the forward reaction \( v_f \) is given by the following equations:

**MECHANISM I**

\[
\frac{1}{v_f} = \frac{K_{G-X}}{V_f(G-X)} + \frac{K_Y}{V_f(Y)} + \frac{1}{V_f}
\]

**MECHANISM II**

\[
\frac{1}{v_f} = \frac{K_{G-X}}{V_f(G-X)} + \frac{K_Y}{V_f(Y)} + \frac{K_{G-X}E_0}{V_f(Y)(G-X)} + \frac{1}{V_f}
\]

The constants of the rate equations are defined in terms of the rate constants of Mechanism I and Mechanism II as follows:

**MECHANISM I**

\[
K_{G-X} = \frac{k_1}{k_4} \quad V_f = \frac{k_4 k_2 E_0}{(k_3 + k_2)} \quad K_Y = \frac{k_3 (k_4 + k_2)}{k_2 (k_3 + k_4)}
\]

**MECHANISM II**

\[
K_{G-X} = \frac{k_3}{k_1} \quad K_Y = \frac{k_4}{k_3} \quad V_f = k_2 E_0
\]

**FIG. 2.** pH optima of hydrolysis and phosphorolysis. O-O, hydrolytic rate of sucrose phosphorylase as a function of pH in 0.05 M Tris-maleate buffer containing 0.08 M sucrose; O---O, phosphorolytic rate of sucrose phosphorylase as a function of pH in 0.05 Tris-maleate buffer containing 0.1 M potassium phosphate and 0.08 M sucrose.

**FIG. 1.** Ultracentrifugation of sucrose phosphorylase. Photographs were taken 9, 25, 41, and 49 min after attainment of 59,780 rpm. The bar angle was 60° and the temperature was 23°. Protein concentration was 7.6 mg per ml in 0.01 M Tris-maleate buffer, pH 7.0.
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Fig. 4 represents \( \frac{l}{v} \) plotted against \( l/glucose-1-P \) at various fructose concentrations. In Fig. 5, results obtained for the reactions \( a-n-glucose-1-P + L-sorbose \rightarrow a-n-glucosyl-a-L-sorbofuranoside + Pi \) are shown. For the three reactions the lines are not parallel at high concentrations of acceptor. At low concentrations of acceptor, the results are consistent with Mechanism I and inconsistent with Mechanism II, which predicts decreasing slopes with increasing concentrations of acceptor. The failure to obtain parallel lines at high concentrations of acceptor suggests that the acceptor competitively inhibits the donor. To account for this inhibition, the following additional equilibrium must be included in Mechanism I:

\[
Y + E \xrightarrow{k_{12}} E \cdot Y
\]

If this additional equilibrium is taken into account and a new constant \( K_{R} = k_{10}/k_{12} \) is introduced, the initial velocity for the forward reaction becomes:

\[
\frac{1}{v_f} = \frac{K_{0-X}}{V_f(G-X)} \left( 1 + \frac{Y}{K_R} \right) + \frac{K_R}{V_f(Y)} + \frac{1}{V_f}
\]

According to this equation, a plot of \( 1/v_f \) against \( 1/G \cdot X \) at various concentrations of \( Y \) should give straight lines with slope \( K_{0-X}/V_f \) (1 + \( Y/K_R \)). The slopes will be independent of \( Y \) at low concentrations, i.e. \( Y/K_R \ll 1 \), and will increase at high concentrations of \( Y \). The slopes of the lines in Figs. 3, 4, and 5 show such a dependence on \( Y \). Equation 11, therefore, is consistent with the observed kinetics of the sucrose phosphorylase reaction.

If the slopes of the lines obtained in Figs. 3, 4, and 5 are plotted against \( Y \) (\( Y = P_i, \) d-fructose, or L-sorbose), lines should be obtained with slope \( K_{R} = k_{10}/k_{12} \) and intercept \( K_{0-X}/V_f \). K\(_R\) can, therefore, be evaluated from such a plot. A plot of this would, therefore, tend to decrease with increasing \( Y \) concentrations and approach \( K_{o,X}/V_f \) at high concentrations of \( Y \).

The kinetics for the reaction \( sucrose + P_i = a-n-glucose-1-P + p-fructose \) was examined in the forward and reverse direction (sucrose, \( P_i, \) \( a-n-glucose-1-P, p-fructose \) correspond to \( G-X, Y, G-Y, \) and \( X \) in Mechanisms I and II). A plot of \( 1/v_f \) against \( 1/sucrose \) at various concentrations of \( P_i \) is shown in Fig. 3. Fig. 4 represents \( 1/v_f \) plotted against \( 1/glucose-1-P \) at various fructose concentrations. In Fig. 5, results obtained for the reactions \( a-n-glucose-1-P + L-sorbose \rightarrow a-n-glucosyl-a-L-sorbofuranoside + Pi \) are shown. For the three reactions the lines are not parallel at high concentrations of acceptor.

Mechanism I incorporates the essential features of the mechanism proposed by Doudoroff: the formation of a glucose-enzyme intermediate and the formation of \( X \) prior to reaction with \( Y \). Mechanism II does not involve formation of a glucose-enzyme intermediate, and the formation of \( G \cdot Y \) and \( X \) occurs concomitantly. The two mechanisms can be distinguished kinetically if \( K_{R,a-X} \) is not small compared to \( K_Y \) and \( K_{o-X} \). If Mechanism I is operative, a plot of \( 1/v_f \) against \( 1/G \cdot X \) at various concentrations of \( Y \) should give a series of parallel lines with slope \( K_{R,a-X}/V_f \) and intercept \( 1/V_f (K_Y/Y) + 1 \). A plot of \( 1/v_f \) against \( 1/Y \) at various concentrations of \( G-X \) should also give parallel lines. For Mechanism II, a plot of \( 1/v_f \) against \( 1/G \cdot X \) at various concentrations of \( Y \) would not give parallel lines since the slope of each line depends on \( Y \). The slope in this case would be \( 1/V_f (K_{o,X} + K_{R,a-X}/Y) \). The slope
The observed rate of hydrolysis was therefore taken as approximately 20\% at the lowest concentration used. The rate of the rate of hydrolysis was observed over the concentration range except for \(\alpha\)-D-glucose-1-P, for which the rate decreases approximately 20\% at the lowest concentration used. The observed rate of hydrolysis was therefore taken as \(V_f\). \(K_{d-X}\) must be less than the lowest concentration of donor used. The values of \(K_{d-X}\) so obtained were confirmed by an alternative method of determination that is discussed below.

To test the quantitative significance of the constants of Table II, these constants were used to calculate the equilibrium constant for the reaction: sucrose + \(P_i\) \(=\) \(\alpha\)-D-glucose-1-P + D-fructose. The equilibrium constant is related to the kinetic constants as follows (22):

\[
K_{eq} = \frac{(\text{sucrose}) \cdot (P_i)}{(\alpha\text{-D-glucose-1-P}) \cdot (\text{D-fructose})}
\]

\[
= \frac{(V_f(\text{sucrose})) \cdot K_{d-X} \cdot K_Y \cdot (P_i)}{(V_f(\text{D-fructose})) \cdot K_Y \cdot (\alpha\text{-D-glucose-1-P})} \cdot K_Y \cdot (\alpha\text{-D-glucose-1-P}) \cdot K_Y \cdot (\text{D-fructose})
\]

\[
= \frac{(0.91 \times 10^3) \cdot (2.5 \times 10^3) \cdot (1.8 \times 10^3)}{(1.4 \times 10^3) \cdot (2.3 \times 10^3) \cdot (13 \times 10^3)} = 0.064
\]

In the above formulation, \(V_f(\text{sucrose}) = V_f\) for \(\alpha\)-D-glucose-1-P, etc. From direct measurements, \(K_{eq} = 0.056 - 0.058\) (at pH 6.6) was determined (23). In view of the number of constants required to calculate \(K_{eq}\), the calculated and experimental values are in good agreement.

It has previously been observed (23) that glucose is an effective inhibitor of sucrose phosphorylase. The kinetics of the inhibition was therefore examined. Two mechanisms of inhibition were considered (22).

1. Glucose combines with the free enzyme and not with glucose-enzyme (E-G). Equation 13 is the rate equation for this type of inhibition (\(v_i = \) inhibited rate, \(K_{d'} = \) dissociation constant for the enzyme-inhibitor complex).

\[
\frac{1}{V_i} = \frac{K_{d-X}}{(G-X)V_f} \left( \frac{G}{K_{d'} + 1} \right) + \frac{K_Y}{(Y)V_f} + \frac{1}{V_f} \tag{13}
\]

2. Glucose can combine both with the free enzyme and glucose-enzyme (E-G). The rate equation for this type of inhibition is:

\[
\frac{1}{V_i} = \frac{K_{d-X} \cdot G}{(G-X)V_f} \left( \frac{G}{K_{d'} + 1} \right) + \frac{K_Y}{(Y)V_f} \left( \frac{G}{K_{d'} + 1} \right) + \frac{1}{V_f} \tag{14}
\]

According to Equation 13, a plot of \(1/v_i\) against \(1/G-X\) at constant \(Y\) and varying concentrations of glucose should give a series of straight lines with common intercepts and slopes dependent on the glucose concentration. If Equation 14 applies, the lines will not have a common intercept. Furthermore, a plot of \(1/v_i\) against \(1/Y\) will give lines with slopes unaffected by the glucose concentration if Equation 13 applies. If Equation 14 applies, the slope of the lines will be a function of glucose concentration. Both types of graphs are shown in Figs. 7 and 8. The results are consistent with Equation 13 and inconsistent with Equation 14. The assumption that glucose combines only with the free enzyme and not with the glucose-enzyme is therefore supported by the kinetic results. From the data of Fig. 7, \(K_{d'} = 4.6 \times 10^{10}\) was determined. Since \(K_{d'}\) is known, an additional method for the determination of \(K_{d-X}\) is available.

\[
K_{d-X} = \frac{4.6 \times 10^{10}}{K_{d'} (1 + (G)/K_{d'}) + (G-X)} \tag{15}
\]

\(K_{d-X}\) can be calculated from the amount of inhibition obtained in the presence of glucose. The ratio of the inhibited velocity (\(v_i\)) to the uninhibited velocity (\(v_0\)) is given by Equation 15:

\[
\frac{v_i}{v_0} = \frac{K_{d-X} + (G-X)}{K_{d-X} (1 + (G)/K_{d'}) + (G-X)}
\]

\(1\) In this equation, inhibition by acceptor is ignored.
where $G =$ glucose concentration. $K_{g-x}$ was calculated from Equation 15 for $\alpha$-d-glucose-1-P, for the reaction with $\alpha$-fructose. $K_{v-x}$ was also determined with the use of Equation 15 for the hydrolysis of $\alpha$-d-glucose-1-P for which the initial velocity method was not applicable and only an estimated value had been obtained. For these inhibition experiments, a reaction mixture containing 0.02 mM $\alpha$-fructose, and 0.01 mM $\alpha$-glucose was used. All other conditions were identical with those used in the determination of the other kinetic constants $v_i/v_0 = 0.26$ was found. From this ratio, $K_{(g-x)}$ for $\alpha$-d-glucose-1-P of $2.3 \times 10^{-4}$ can be calculated, which is in agreement with a value obtained by other methods, given in Table II. Under identical conditions of reaction, except that $\alpha$-fructose was omitted, $K_{(g-x)}$ for the hydrolysis of $\alpha$-d-glucose-1-P was determined. $v_i/v_0 = 0.58$ was found from which $K_{g-x} = 0.67 \times 10^{-4}$ was calculated. This is somewhat higher than the estimated value of $0.46 \times 10^{-4}$ given in Table II. However, it confirms that the $K_{g-x}$ for the hydrolytic reaction is lower than that obtained with other acceptors.

Direct Detection of Glucose-Enzyme Complex (24)—Incubation of the enzyme with substrate in the absence of a glucosyl acceptor should lead to the accumulation of an enzyme-glucose complex. Because of the hydrolytic reaction, we have so far been unable to isolate this complex in a functional form. However, we were able to isolate a denatured form of the complex by adding the enzyme, after incubation with sucrose-$^{14}$C (uniformly labeled), to hot methanol. The precipitated denatured protein so obtained was washed extensively and found to contain radioactivity. The results of a typical experiment are shown in Experiment 1, Table III. The experiment was repeated with fructose-labeled sucrose instead of with uniformly labeled sucrose (Experiment 2). Under these conditions, essentially no radioactivity was found in the protein that was precipitated. This shows that the radioactivity found in Experiment 1 is not due to nonspecifically absorbed sucrose, and that only the glucose moiety, and not the fructose moiety, of sucrose is incorporated into the protein. From the amount of glucose bound to the protein, a minimum molecular weight of 80,000

![Double reciprocal plot of initial velocity against sucrose concentration at a fixed $P_i$ concentration and a series of $d$-glucose concentrations.](image1.png)

![Double reciprocal plot of initial velocity against $P_i$ concentration.](image2.png)

**Table III**

| Amount of glucose bound to denatured sucrose phosphorylase after reaction with sucrose |

The reaction mixture contained: enzyme, 0.8 to 0.9 mg; Tris-maleate buffer (pH 7.0), $3 \times 10^{-3}$ M; sucrose-$^{14}$C, $1.4 \times 10^{-3}$ M; final volume, 0.16 ml. The reaction was carried out at $25^\circ$. After 15 sec, the reaction mixture was added to 3 ml of boiling methanol. Acetone, 1.0 ml, was then added. The enzyme that was precipitated was washed eight times with 5.0 ml of methanol and then three times with 2 ml of 0.01 M $K^+PO_4^-$ buffer (pH 7.0) containing 0.01 M sucrose, 0.01 M $d$-fructose, and 0.01 M $d$-glucose. One-half of the precipitate was then dissolved in 0.5 ml of 1 M NaOH. Aliquots of this solution were used for the determination of the protein by the method of Lowry et al. Under these conditions, essentially no radioactivity was found in the protein that was precipitated. In no case did the additional washing change the specific activity by more than 15%; in most experiments it had no effect.

<table>
<thead>
<tr>
<th>Additions to reactions</th>
<th>Activity $cpm/mg\ protein \times 10^4$</th>
<th>$\mu$moles/mg\ protein $\times 10^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1: sucrose-$^{14}$C (uniformly labeled); specific activity, $1.1 \times 10^3$ cpm per $\mu$ mole</td>
<td>7.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Experiment 2: sucrose labeled in the $d$-fructose moiety; specific activity $1.1 \times 10^3$ cpm per $\mu$ mole</td>
<td>0.048</td>
<td>0.004</td>
</tr>
<tr>
<td>Experiment 3: sucrose-$^{14}$C (uniformly labeled) and $d$-glucose, $3.3 \times 10^{-3}$ M</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Experiment 4: sucrose-$^{14}$C (uniformly labeled) and $d$-fructose, $6.3 \times 10^{-3}$ M</td>
<td>1.1</td>
<td>0.19</td>
</tr>
</tbody>
</table>
can be calculated. This is in good agreement with a molecular weight of 80,000 to 100,000 estimated from the sedimentation constant and the elution volume from Sephadex G-200 columns.

Since the hydrolytic reaction (4) catalyzed by sucrose phosphorylase leads to the formation of \( n \)-glucose, the possibility was considered that the glucosyl-enzyme was the result of the interaction of the protein with free \( n \)-glucose. Sucrose-\( ^{14} \)C (uniformly labeled) was, therefore, added to the enzyme in the presence of unlabeled glucose. Experiment 3 in Table III shows that this did not lead to a decrease in the radioactivity associated with the protein. The formation of the glucose-enzyme must have occurred therefore by reaction with sucrose and not with free glucose.

If the glucosyl-enzyme isolated in these experiments is an intermediate in the reactions catalyzed by sucrose phosphorylase, then the presence of a glucosyl acceptor should lower the steady state concentration of glucosyl-enzyme. This would lead to a decrease in the radioactivity of the isolated denatured protein. To establish the effect of a glucosyl acceptor, sucrose-\( ^{14} \)C (uniformly labeled) was reacted with the enzyme in the presence of \( n \)-fructose. Experiment 4 (Table III) shows that the amount of glucose-enzyme was reduced by approximately 80%.

The experiments with uniformly labeled sucrose and \( n \)-fructose-labeled sucrose establish that only the \( n \)-glucose moiety of sucrose is associated with the protein. As a first step in establishing the nature of the interaction between the \( n \)-glucose moiety of sucrose and the protein, we attempted to dissociate the radioactive compound from the precipitated complex and identify the released radioactive compound. As previously pointed out, extensive washing at neutral pH did not release the protein-bound radioactivity. However, treatment of the precipitated protein with dilute base released the protein-bound radioactivity. About 0.2 mg of washed glucose-enzyme complex was suspended in 0.5 ml of 0.01 M NaOH for 2 hours. The suspension was then neutralized and filtered through a Millipore filter and the filtrate was lyophilized. The lyophilized filtrate was dissolved in 0.2 ml of water and placed on a Sephadex G-50 column with a 10-ml exclusion volume. The radioactivity peak obtained from this column was lyophilized and chromatographed on Whatman No. 1 paper with \( n \)-butyl alcohol-pyridine-water (6:4:3) and \( n \)-butyl alcohol-acetic acid-water (4:1:5) (top layer) as developing solvents. In both solvent systems there was only one radioactive peak, which migrated with the same \( R_f \) as \( n \)-glucose. In a similar experiment, after incubation for 10 min in 0.01 M NaOH, 60% of the radioactivity bound to the protein was released. These results show that the denatured complex contains \( n \)-glucose bound in such a way that it is released upon alkaline hydrolysis, or it may possibly contain glucose per se, which is liberated under alkaline conditions. The experiments exclude the possibility that the complex contains some other glucosyl such as \( a \)-\( n \)-glucose-1-P which might have been produced by reaction of sucrose with traces of \( P_i \) present in the reaction mixture.

**Discussion**

Two new facts have been presented which support the mechanism of action of sucrose phosphorylase originally proposed by Doudoroff et al. (1). It has been shown that the kinetics of the reaction are consistent with a double displacement mechanism (Mechanism I) and not consistent with a single displacement (Mechanism II). Direct evidence for the occurrence of a glucose-enzyme intermediate was derived from the isolation of a denatured glucose-enzyme complex. To show that a substrate-enzyme complex is, in fact, an intermediate in the overall catalytic process, it is desirable to show that this complex can give rise to products at a rate consistent with the catalytic reaction. Since it was necessary to denature the complex, this criterion could not be applied. The following facts, however, show that the isolated complex was derived from an intermediate involved in the catalytic process.

1. The isolated complex contained the \( n \)-glucose moiety of sucrose but not the \( n \)-fructose moiety, and the possibility that the complex was formed from free glucose and enzyme was eliminated. Consequently, the isolated enzyme must have been derived from an intermediate which was formed after the enzyme had reacted with sucrose to break or labilize the \( n \)-glucose-\( n \)-fructose bond.

2. According to Mechanism I, addition of \( X \) (i.e. \( n \)-fructose if \( G-X = sucrose \)) should lower the steady state concentration of \( G-E \), and reduce the rate of formation of \( G-Y \). If the denatured complex is derived from \( G-E \), then the amount of complex which can be isolated should be lowered by the addition of \( n \)-fructose. When fructose and sucrose-\( ^{14} \)C were simultaneously added to the enzyme, the amount of glucose-enzyme complex which could be isolated was reduced by 80% (Experiment 4, Table III).

3. In the absence of \( Y \), the formation of the glucose-enzyme complex is an essentially irreversible process. Therefore, the addition of a competitive inhibitor for \( G-X \) will not reduce the concentration of the glucose-enzyme complex, although it will reduce the level of reversible complexes formed prior to formation of the glucose-enzyme complex. The addition of \( n \)-glucose, a competitive inhibitor, did not reduce the amount of isolatable glucose-enzyme complex (Experiment 3, Table III).

At this stage, no conclusions can be reached regarding the nature of the chemical bond between glucose and the denatured protein. This bond need not necessarily be a covalent bond. It is possible that in the insoluble complex isolated glucose is strongly absorbed to the protein. If this is the case, then the glucose must have been formed on the enzyme during the process of denaturation, since it has been shown that the complex could not have been formed by the reaction of enzyme and free \( n \)-glucose. It should also be pointed out that if the isolated complex contains covalently bound \( n \)-glucose, the nature of the bond and locus of binding is not necessarily the same as that in the enzymatically active glucose-enzyme complex.

In addition to providing evidence for the occurrence of the glucose-enzyme complex, the kinetic data also lead to some tentative conclusions concerning the mode of binding of the substrate to the enzyme. The data indicate that the major contribution to the binding of the substrate comes from the glucose moiety of the substrate. The substrate (\( G-X \)) for sucrose phosphorylase consist of two components: the \( n \)-glucose moiety (\( G \)), which all substrates contain, and a variable component (\( X \)), which can be a carbohydrate or \( P_i \). The inhibition data provide a measure of the extent of binding of each of these.

\( 2 \) In recent experiments, we have been able to denature the glucose-enzyme complex without precipitating the protein (Voet and Abeles, unpublished results). The binding of \( n \)-glucose to the protein is therefore not dependent upon converting the protein to an insoluble form.
components to the substrate-binding site. The dissociation constant for the inhibited glucose-enzyme complex, \( K_d = 4.6 \times 10^{-4} \), and the corresponding \( K_i \) for \( \alpha\)-fructose or Pi = \( 2.5 \times 10^{-4} \) indicate that the affinity of \( \alpha\)-glucose for the substrate-binding site is 500 times greater than that of fructose or Pi. These results suggest that the binding of the substrate to the enzyme is almost entirely due to the \( \alpha\)-glucose moiety of the substrate. If \( K_d \cdot X \) obtained for \( \alpha\)-n-glucose-1-P and sucrose with various acceptors (except \( H_2O \)) is a dissociation, the substrates \( \alpha\)-n-glucose-1-P and sucrose are less tightly bound than glucose, so that the \( X \) moiety makes a negative contribution to the binding of the substrate. Nevertheless, specificity studies show that the \( X \) moiety is important in determining which disaccharides can function as substrates (25). These observations can be explained in terms of the “induced fit” theory (26). The \( X \) moiety, although it contributes nothing to the binding of the substrate, interacts with the protein so that it assumes the proper conformation for catalytic activity.

REFERENCES

\(^{2}k_{d-x}\) is a dissociation constant for the substrate enzyme complex only if \( k_2 \) is the rate-limiting step. Since \( V_f \) for any substrate and \( k_{d-x} \) for all acceptors, except \( H_2O \), are of the same order of magnitude, this may indeed be the case with all acceptors other than \( H_2O \). In the hydrolytic reaction, another step, possibly the reaction of \( H_2O \) with the glucose-enzyme complex, may be rate limiting. At this stage, insufficient information is available to decide conclusively which step is rate limiting.

Purification and Mechanism of Action of Sucrose Phosphorylase
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