Glucose-1-Phosphate: Enzymatic Formation from Starch

THEORY

Starch, a carbohydrate found in most plants, is a mixture of two polysaccharides: amylose, a straight-chain polymer of glucose units joined by α-1,4 linkages, and amylopectin, a branched-chain glucose polymer that differs from glycogen primarily in its larger number of α-1,4-linked glucose units between the α-1,6 branch points. Both glycogen and starch are acted upon by the enzyme phosphorylase, which catalyzes the phosphorolysis of glycogen or starch to glucose-1-phosphate (see Figure 15-1).

The primary function of starch is to serve as a storage form for carbohydrate. Mobilization of starch for metabolism or synthesis of other polysaccharides requires cleavage of monosaccharide units from the polymer and formation of phosphorylated sugar derivatives, which are the substrates for glycolysis or for formation of nucleoside diphosphosugars. Phosphorolysis of starch fulfills both requirements in a single step. If starch were cleaved by hydrolytic reactions (e.g., by amylases), subsequent formation of the sugar phosphate from the glucose released would require the expenditure of metabolic energy in the form of

ATP. Thus, the phosphorylase reaction increases the efficiency of starch utilization by conserving the energy of the acetal links between glucose units through formation of a phosphoacetal linkage.

The regulation of glycogen phosphorylase in mammalian muscle and liver has been intensively studied. As might be expected for a pathway governing use of a major energy reserve, this enzyme (and the corresponding synthetic enzyme, glycogen synthetase) is under complex metabolic and hormonal control. Students should consult a textbook for details.

In this experiment, soluble starch is incubated with phosphate and a phosphorylase preparation from potatoes. After 24–48 hrs, the reaction is stopped by heating of the mixture to destroy the enzyme. After removal of the denatured enzyme, the unreacted inorganic phosphate is removed from the filtrate by precipitation as magnesium ammonium phosphate. The glucose-1-phosphate is then isolated by ion-exchange chromatography and is subsequently crystallized.

Glucose-1-phosphate is determined by measurement of the inorganic phosphate released by acid hydrolysis. Phosphoacetals of all sugars can be hy-
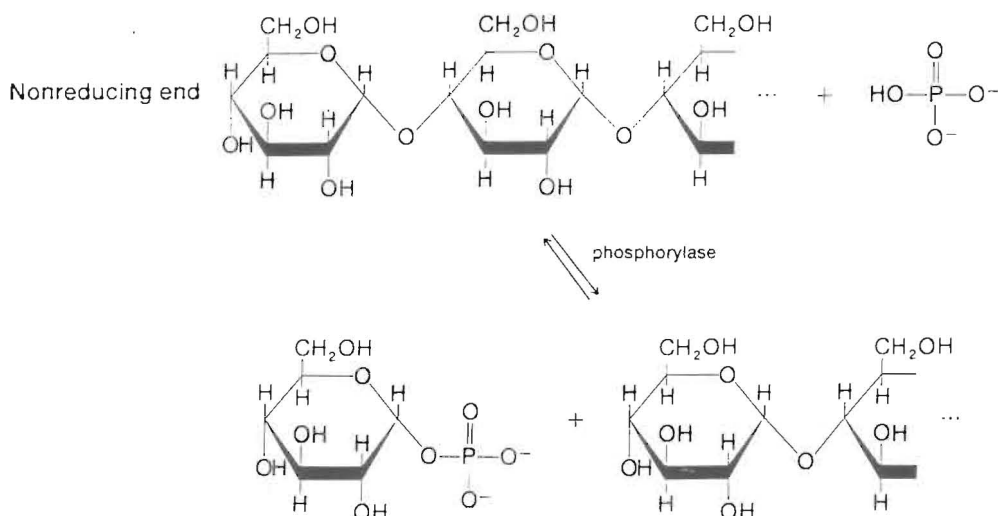
FIGURE 15-
Phosphorylase-catalyzed phosphorolysis of glycogen or starch to glucose-1-phosphate

drolyzed by treatment with 1 N acid for 7 min at 100°C, but the various sugar phosphate esters (i.e., glucose-6-phosphate) ordinarily require longer heating times or more concentrated acid for complete hydrolysis.

Inorganic phosphate is measured by the colorimetric method of Fiske and Subbarow (1925), which is specific for inorganic phosphate; i.e., inorganic phosphate can be analyzed in the presence of organic phosphates such as phosphate esters or phosphoacetals. Hence, the increase in inorganic phosphate after 7-min hydrolysis of a sample (over an unhydrolyzed blank) is a measure of the phosphoacetal content of the sample. Stability of various other biologically important phosphate compounds to acid hydrolysis has been discussed in the introduction to this section (p. 144). The color developed in the Fiske-Subbarow reaction is dependent on the formation of a phosphomolybdic acid complex, which forms an intense blue color when reduced by a mixture of bisulfite and p-methylaminophenol (Elon).

EXPERIMENTAL PROCEDURE

Materials

Phenylmercuric nitrate slurry
0.8 M Potassium phosphate buffer pH 6.7
Soluble starch
Filter aid
14% NH_4OH
2 N NaOH
$Mg(OAc)_2 \cdot 4H_2O$
IR-45 (OH^- form)
Absolute methanol
Cheesecloth
Cylindrical glass tubes
Acid molybdate reagent
Phosphate standard (1 mM)
2 N HCl
Potatoes
Dowex 50 (H^+ form)
5% KOH
Blender
Glass wool
Charcoal
Reducing reagent (3% $NaHSO_3$,
 1% p-methylaminophenol)
0.01 N KI, 0.01 N I_2
Centrifuges
300 ml bottles
Vacuum desiccator
P_2O_5

General

The preparation and isolation of glucose-1-phosphate can be completed within three laboratory periods. The enzyme incubation can be started in the first period. The removal of cations with Dowex 50 should be completed in the second period and the product stored at 0°–5°C. The ion exchange can then be completed and the crystallization begun in the third period. Experiment 16 may be started in the fourth period, after the collecting and drying of the crystals of dipotassium glucose-1-phosphate dihydrate. Deionized or distilled water should be used throughout, and no solutions should be discarded until a product is obtained. Store

all solutions at 0°–5°C between laboratory periods to avoid bacterial or chemical degradation.

Preparation of Starch

Using a minimum of H_2O, make a smooth slurry of 10 g of soluble starch. Add this to 70 ml of vigorously boiling H_2O, and stir until the solution is nearly clear. The solution may be cloudy, but should not be milky. Further heating may be required to dissolve the starch, but avoid prolonged heating. Add 180 ml of cold H_2O to help cool the solution to room temperature. Do not add the enzyme until the solution has cooled to room temperature (heat inactivates the enzyme).

Preparation of Enzyme

Cut a medium-sized potato (precooled for 24 hrs at 1°–5°C; you need not peel the potato) into half-inch cubes. Blend 150 g of these cubes, added over a 30-sec period, with 150 ml of H_2O for 2 min in a blender. Then quickly pour the resultant slurry onto a Buchner funnel lined with 2–4 layers of cheesecloth, and filter with vacuum, washing the crude pulp with 25 ml of H_2O to insure thorough enzyme extraction. Failure to complete these operations within 2 min of blending may result in loss of enzyme activity. After filtration, stir in 100 mg of phenylmercuric nitrate, as a powder or slurry, to inhibit the action of other enzymes and bacterial growth. Allow the preparation to stand for 1 min; then decant the solution and any floating phenylmercuric nitrate from the accumulated precipitate. Adjust the extract to a volume of 250 ml with H_2O. The cooled starch solution should be mixed with the potato enzyme extact *immediately* after the extract is prepared.

Incubation of the Enzyme with Starch

Add the 250 ml of enzyme solution to 250 ml of starch solution. Then add 250 ml of 0.8 M phosphate buffer solution, record the total volume, and store the solution in a stoppered Erlenmeyer flask in your desk.

During the incubation period (24–48 hrs) the reaction mixture will turn red and then dark blue or purple because of the action of other enzymes present in the crude extract. The colored materials will be removed during later procedures.

Removal of Inorganic Phosphate

After 24–48 hrs of incubation, stop the enzymatic reaction by rapidly heating the solution to 95°C and then slowly cooling it over a 30-min period. If possible, come to the laboratory 4–6 hrs before the usual labora-

tory period to carry out the heating, so that the solution will slowly cool before the next step. Remove the coagulated protein by centrifuging the fluid in large bottles for 15 min at 4,000 × g. Decant the supernatant fluid into a large beaker. Then remove the excess phosphate by dissolving 0.2 mole of magnesium acetate (44 g of $Mg(OAc)_2 \cdot 4H_2O$) in the solution and adjusting the pH to 8.5 with 14% ammonia (use pH paper first, then a pH meter; about 30 ml of 14% NH_4OH will be required, but avoid adding excess NH_4OH). Cool the solution in a salted ice bath for 10 min, and remove the precipitated magnesium ammonium phosphate by suction filtration. Use filter paper covered with a thin layer of celite filter aid. If filtration is very slow, use more than one Buchner funnel or change the filter. Record the volume of the filtrate. Remove duplicate 0.05, 0.1, 0.2, and 0.5 ml aliquots of the filtered solution for the inorganic phosphate and 7-min phosphate assays described later. If the phosphate assay reveals that an excess of inorganic phosphate is still present in the incubation filtrate (i.e., if an intense blue color forms in the unhydrolyzed 0.1 ml aliquot), add 1 g of $Mg(OAc)_2 \cdot 4H_2O$, adjust to pH 8.5 with 14% ammonia, cool the solution, and filter it again before repeating the phosphate assays. Calculate the number of μmoles of inorganic phosphate and 7-min phosphate in the entire volume of filtered solution. If the inorganic phosphate in the filtrate is less than 15% of the phosphate found after 7 min hydrolysis, you may proceed with the rest of the experiment.

Assay of Inorganic Phosphate and Glucose-1-Phosphate. Range Finding for Phosphate Assay

In the Fiske-Subbarow colorimetric method for the determination of phosphate, the color yield is directly proportional to the amount of inorganic phosphate only when the aliquot taken for analysis contains between 0.1 and 1.0 μmole of phosphate. The efficiency of the enzymatic formation of glucose-1-phosphate will vary somewhat depending upon the sources of the enzyme and the starch as well as the length of the incubation period. Accordingly, the aliquot sizes suggested may not lie within the range in which the assay is valid. In this event, analyze several different aliquot sizes until you find one that falls within the accurate range of the assay. In this experiment, as in all isolation procedures, you must obtain an accurate measurement

of the amount of the desired compound in each fraction. Therefore, you will need to determine and use appropriate aliquot ranges for analysis before going to the next steps in the experiment. Further, you must keep accurate records of aliquot sizes and protocols in order to evaluate your data correctly.

Most laboratory detergents contain large amounts of phosphate, which may contaminate your glassware. All tubes used in phosphate analyses should be thoroughly cleaned and rinsed with deionized water.

7-Min Hydrolysis

Set aside one of the duplicates of each pair of samples to be assayed for inorganic phosphate. To the other sample add an equal volume of 2 N HCl; 1 ml of 1 N HCl is preferable with aliquots of less than 0.2 ml. Now place the acidified samples in a boiling water bath for 7 min. Remove the samples, cool and neutralize them to pH 6.5–7.5 by adding (pipette) a stoichiometric amount of 2.0 N NaOH. It is important that the solutions be nearly neutral; use pH paper to check them. Dilute all the tubes (including the unhydrolyzed aliquots) to 3 ml with H_2O.

Inorganic Phosphate Determination (Modified Fiske-Subbarow Method)

For each phosphate analysis prepare tubes containing a water blank, unhydrolyzed aliquots, hydrolyzed aliquots, and phosphate standards (0.1, 0.2, 0.4, 0.6, 0.8, and 1 μmoles of inorganic phosphate). Adjust the volume of all tubes to 3.0 ml with water, and add, in order, 1 ml of acid molybdate reagent, 1 ml of reducing reagent (3% $NaHSO_3$, 1% Elon), 5 ml of H_2O. Mix the solutions by inverting the tubes and allow the color to develop for 20 min before reading the absorbance at 660 nm. Calculate the quantity of inorganic phosphate and glucose-1-phosphate in the aliquot and in the entire reaction mixture. In subsequent phosphate analyses you may use the standard curve obtained during your first analysis, if you include one or two standards (0.4 and 0.8 μmoles) to insure that the color yield is constant.

Use of Cation-Exchange Resin

Decolorize the solution of glucose-1-phosphate (freed of inorganic phosphate) by stirring with 2 g of charcoal and then removing the charcoal by vacuum filtration using filter aid. This procedure yields a clear or yellowish solution containing glucose-1-phosphate, unreacted starch, and many salts that were present either in the original potato extract or in the reagents added during the course of the experiment.

Remove the cations by treatment with Dowex 50 in the following manner: Add 350 ml of moist Dowex 50 in H^+ form to the decolorized solution and stir gently for 5 min before separating by vacuum filtration. Do not use filter aid when removing the Dowex. Return the Dowex at the end of the period. (Note: Do NOT allow the Dowex to become mixed with the Amberlite resin used in the next step!) If the pH of the filtrate is not acidic (pH 1.0–3.0), add 100 ml of moist Dowex 50 in H^+ form, then stir and filter the solution as before. Repeat this procedure until the pH of the solution is 1.0–3.0. Record the volume of the resultant solution, and remove duplicate aliquots identical to those previously used in the assay of the $MgNH_4PO_4$ supernate. Assay these for inorganic phosphate and 7-min phosphate. If necessary, store the remaining solution at 0°–5°C for several days. There may be small losses of glucose-1-phosphate during storage in acidic solution. These are fewest at 0–5°C. Alternatively, if time is sufficient, proceed directly to the next step.

Use of Anion-Exchange Resin

The next step in the purification procedure is the column chromatography of the Dowex 50 filtrate on Amberlite IR-45 in OH^- form. This involves removing the anions in the acid solution from all other contaminating materials. Thus, when the acidic filtrate (pH 1–3) contacts the IR-45 in OH^- form, the glucose-1-phosphate is ionically adsorbed on the resin while unionized materials in the acid solution, such as acetic acid and unreacted starch, pass through the column. When the resin is eluted with strong alkali (5% KOH), the adhering anions are displaced by the OH^- ions and are obtained in the eluate from the column.

Prepare a column about 4 cm in diameter and 20–30 cm long using a rubber stopper, screw clamp, and glass wool plug (Figure 15-2). Mix 250 ml of H_2O with 250 ml of moist IR-45 (OH^-), and pour the resultant slurry into the column so that there are no air pockets in the settled resin. Then wash the resin with H_2O until the effluent is about pH 9.0 or lower (pH meter). Drain or pipette off the excess fluid until the fluid surface just covers the top of the resin bed. Cover the surface of the resin with a layer of glass wool.

To cause adsorption of the glucose-1-phosphate by the IR-45, gently pour the acidic solution treated with Dowex 50 onto the column, avoiding the introduction of air bubbles into the resin bed. Open the screw clamp, and adjust the flow rate to about 15 ml/min. Pass the entire solution through the IR-45 resin without permitting air to enter the column. Collect the entire effluent, determine the volume, and assay duplicate aliquots for inorganic phosphate and 7-min phosphate.
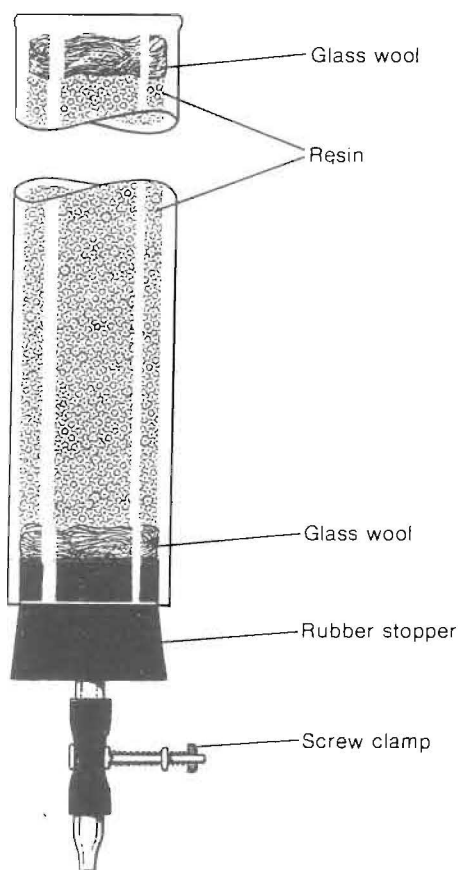
FIGURE 15-2
Anion exchange column.

To elute the glucose-1-phosphate from the resin, pass 5% KOH through the column, adjusting the flow rate to 20 ml/min. Collect separate, successive 100 ml fractions in a graduate. Continue collecting fractions for about 500 ml after the pH of the effluent becomes markedly alkaline (pH 11.0–13.0) as observed with pH paper. (The usual total volume of all fractions equals 700–800 ml.) Then test 0.1 ml aliquots from each fraction for 7-min phosphate. Some fractions require smaller aliquots to fall within the linear range of the standard curve, but 0.1 ml aliquots will serve to find the "peak" for glucose-1-phosphate. Combine the fractions containing 80–90% of the recovered glucose-1-phosphate, and adjust the solution to pH 8.0 or higher by adding a few drops of 5% KOH, if necessary. Determine the volume of the combined KOH effluent (glucose-1-phosphate "peak") fractions. Save duplicate 0.05, 0.1, 0.2, and 0.5 ml aliquots for inorganic phosphate and 7-min phosphate analysis.

Add 3 vol of absolute methanol to the combined fractions, and leave them at 0°–5°C for at least 12 hrs for crystallization of dipotassium glucose-1-phosphate dihydrate. In this temperature range this compound will remain stable indefinitely. Finally, collect the crystals by centrifugation, after pouring off the bulk of the clear supernatant fluid. Wash the crystals with 5 ml of absolute ethanol, centrifuge again, then dry them in a tared container in a vacuum desiccator over P_2O_5 at 5°C. Weigh the crystals to determine the final yield of glucose-1-phosphate and save them for analysis in Experiment 16.

Report of Results

1. Prepare a flow sheet of the steps in the isolation procedure, indicating the purpose of each step.

2. Prepare a table (see Table 15-1) showing the percentage of the glucose-1-phosphate recovered at each step in the procedure. Account for any poor recoveries.

If appreciable 7-min phosphate appears (>30% of G-1-P of Dowex 50 filtrate), and if time permits, add 50 g of IR-45 to the column, and pass the effluent through the column again. After the original solution has passed into the column and the effluent has been satisfactorily freed of the 7-min phosphate, wash the column with deionized H_2O until the effluent no longer gives a positive test for starch (i.e., a blue color upon mixing a drop of effluent with a drop of 0.01 N I_2 in 0.01 N KI). About 500 ml of water will be required to remove all of the starch from the column.

TABLE 15-1
Percentage of Glucose-1-Phosphate Recovered During Purification

Step	Vol of solution	μmoles of 7-min phosphate/ml	Total μmoles of 7-min phosphate	Percent recovered
MgNH$_4$PO$_4$ supernatant				100
Dowex 50 supernatant				
Original IR-45 (OH⁻) washes (before eluting with H$_2$O or KOH)				
Combined KOH effluent fractions				
Glucose-1-phosphate crystals*				

*Assume that the crystals are dipotassium glucose-1-phosphate dihydrate (MW = 372).

3. Prepare a graph of the elution pattern of the glucose-1-phosphate, plotting μmoles of glucose-1-phosphate/100 ml as the ordinate and the fraction numbers, or ml of effluent, as the abscissa.

4. Calculate the maximum yield of glucose-1-phosphate that would be expected if the reaction had reached equilibrium. The quantity of starch is altered only slightly during the incubation; therefore, the starch concentrations in the numerator and denominator are roughly equivalent and cancel out. Use as your equilibrium constant for calculation

$$K_{eq} = \frac{\text{Glucose-1-phosphate}}{\text{Inorganic phosphate}} = 0.088$$

Remember that the initial concentration of inorganic phosphate (0.8 M $\times$ 250 ml/750 ml = 0.267 M) has been decreased at equilibrium by the amount of glucose-1-phosphate formed. Compare your yield (glucose-1-phosphate found in the $MgNH_4PO_4$ supernatant fluid) with the theoretical yield expected. Suggest reasons for any discrepancy between your results and the theoretical value calculated from the phosphorylase constant. Comment on the recovery of the various steps of the purification procedure.

EXERCISES

1. Consider the following set of data for inorganic phosphate determinations on unhydrolyzed and 7-min hydrolyzed aliquots taken from a 700 ml volume of the $MgNH_4PO_4$ supernatant obtained in this experiment.

a. Which absorbance values can be used for further calculations?

b. Assume 100% recovery of the glucose-1-phosphate and inorganic phosphate at the beginning of

Vol of aliquot	Absorbance at 660 nm	
	Unhydrolyzed	7-min Hydrolyzed
0.1 ml	0.004	0.225
0.2 ml	0.010	0.435
0.5 ml	0.022	0.860
Standards		
0.4 μmole inorganic phosphate		0.150
0.8 μmole inorganic phosphate		0.300

the anion exchange step. How many milliequivalents of anion exchange resin must be used to handle a pH 3.0 solution of these anions ($pK_{a_1} = 1$, and $pK_{a_2} = 6$, for glucose-1-phosphate)?

c. Assume 80% recovery of the glucose-1-phosphate as crystals. How many grams of dipotassium glucose-1-phosphate dihydrate would be isolated?

2. Propose a series of steps using ion exchange resins for the isolation of glucose-6-phosphate from a solution containing methylamine, sodium acetate, glucose and glucose-6-phosphate.

3. What steps would you perform to convert the rather insoluble barium salt of glucose-6-phosphate to the dipotassium salt?

REFERENCES

Fiske, C. H., and Y. Subbarow. 1925. The Colorimetric Determination of Phosphorus. *J. Biol. Chem.* 66:375.

McCready, R. M., and W. Z. Hassid. 1944. The Preparation and Purification of Glucose-1-Phosphate. *J. Am. Chem. Soc.* 66:560.

McCready, R. M., and W. Z. Hassid. 1957. Preparation of α-D-Glucose-1-Phosphate. *Preparation and Assay of Substrates* (Methods in Enzymology, Vol. 3). New York: Academic Press.

Glucose-1-Phosphate: Chemical Characterization

THEORY

It is necessary to establish the identity of any newly isolated compound. In this experiment, the first step consists of showing that the product presumed to be glucose-1-phosphate is in fact composed of equal quantities of glucose and phosphate. Second, it is also necessary to distinguish between the various isomers of glucose-phosphate. The second step is particularly pertinent to Experiment 16 because we know that glucose-1-phosphate may be converted to glucose-6-phosphate by the enzyme phosphoglucomutase, which is present in the crude potato extracts of Experiment 15.

The characterization of glucose-1-phosphate is based on the relative stability of various sugar phosphates in acid solution. Sugar phosphoacetals are non-reducing sugars, and they release equal amounts of reducing sugar (characterizable by chromatography) and phosphate upon 7-min hydrolysis in 1 N acid at 100°C. In contrast, glucose-6-phosphate and other phosphate esters require more concentrated acid or more prolonged heating for complete hydrolysis. Therefore, it is possible to characterize glucose-1-phosphate by qualitative (chromatographic) and quantitative analysis of materials present before and after 7-min hydrolysis. It is also possible to estimate the purity of the product by careful evaluation of the results.

Chromatographic characterization of sugar phosphates can be achieved in two different ways. First, you can usually detect the sugar portion of sugar phosphates by using sugar-specific reagents, such as the aniline-acid-oxalate or p-anisidine spray reagents (see Experiment 13). Organic phosphates, including sugars phosphates, can also be detected with phosphate specific methods. The modified Hanes-Ischerwood spray reagent used in this experiment hydrolyzes any organic phosphate so that inorganic phosphate is released. The inorganic phosphate then combines with the molybdic acid and is reduced to yield a blue spot (phosphomolybdous acid). The alternate iron-sulfosalicylic acid complex assay features binding of Fe^{+3} by organic phosphates. The bound Fe^{+3} cannot then form a red-brown complex with sulfosalicylic acid.

It is convenient to begin the experiment by preparing the chromatograms and starting chromatographic

development. Then you should perform the phosphate and reducing sugar analyses as described. Finally, spray the developed chromatograms to detect the separated products.

EXPERIMENTAL PROCEDURE

Materials

Nelson's reagents A and B
Glucose standard
1 N NaOH
Reducing reagent
Acid-FeCl₃ in Acetone
1% Glucose-1-phosphate
1% Glucose
UV lamp
Modified Hanes-Ischerwood spray reagent,
   i.e., 4%(NH₄)₆Mo₇O₂₄ · 4H₂O : 1N HCl : 70% HClO₄ :
   H₂O (5 : 2 : 1 : 12)
1.25% Sulfosalicylic acid in acetone
Arsenomolybdate reagent
1N HCl
Phosphate (Pᵢ) standard (1 μmole/ml)
Acid molybdate reagent
100°C oven
Whatman No. 1 paper
1% p-Anisidine spray reagent
1% Glucose-6-phosphate
Methanol
88% Formic acid
10% Mg(NO₃)₂ in ethanol

Characterization of Compounds Present Before and After Hydrolysis

You can check a sample of the suspected glucose-1-phosphate for purity by evaluating the following quantities (tubes with prime values are duplicates; instructions for preparing tubes follow list):

1. reducing equivalents before 7-min hydrolysis (tube 1 and 1')

2. reducing equivalents after 7-min hydrolysis (tubes 2 and 2')

3. inorganic phosphate present before 7-min hydrolysis (tube 3 and 3')

4. inorganic phosphate present after 7-min hydrolysis (tubes 4 and 4')

5. phosphate present after total hydrolysis (tubes 5 and 5')

6. chromotographic characterization after hydrolysis (tube 6)

To determine these values, prepare a 10 ml solution of the isolated, suspected glucose-1-phosphate (1.60–

2.20 mg/ml to two decimal places), and place 0.1 ml aliquots in each of 10 tubes. Number the tubes 1, 1', 2, 2', 3, 3', 4, 4', 5, and 5'. To another tube, Tube 6, add 0.1 ml of a 10 mg/ml solution of isolated glucose-1-phosphate and add 0.1 ml 2 N HCl.

7-Min Hydrolysis. Add 1 ml of 1 N HCl to tubes 2, 2', 4, and 4'. Heat them for 7–8 min in a boiling water bath; then cool them at once in a beaker of cold water. Finally, neutralize the contents by adding 1 ml of 1 N NaOH to each of the tubes. At the same time as the other tubes, heat tube 6 at 100° C, but do not add additional HCl or NaOH to this tube. Cool tube 6 with the other tubes and save the contents for chromatographic analysis.

Total Hydrolysis. Add 1 drop of 10% Mg(NO₃)₂ in ethanol to tubes 5 and 5'. Take the fluid to dryness, cautiously, over a flame until brown fumes disappear and only a white ash remains in the tubes. Cool the tubes, then add 1 ml 1N HCl and heat in a boiling water bath for 15 min to hydrolyze any pyrophosphates that may have formed during the heating with Mg(NO₃)₂. Cool the tubes, neutralize with 1 ml 1 NaOH, and analyze for inorganic phosphate as with the other tubes.

Preparation for Assay of Unhydrolyzed Product. Add 2 ml of H₂O to tubes 1, 1', 3, and 3' and save for the assay of unhydrolyzed product.

Nelson's Test for Equivalents of Reducing Sugar. Analyze tubes 1 and 1' (untreated) and tubes 2 and 2' (hydrolyzed 7 min) by Nelson's test as follows: Prepare a blank sample containing 2 ml H₂O and standards containing 0.1, 0.2, 0.4, 0.5 and 0.8 μmoles of glucose in 2-ml final volume. Mix 0.5 ml Nelson's reagent B with 12.5 ml of Nelson's reagent A. Add 1 ml of the combined reagent to each tube (i.e., the blank, the standards, and 1, 1', 2, and 2'). Place the tubes simultaneously in a vigorously boiling water bath (500 ml beaker or larger), and heat for exactly 20 min. Remove the tubes simultaneously and place them in a beaker of cold water to cool. When the tubes are cool (25° C), add 1 ml of arsenomolybdate reagent to each and shake well occasionally during a 5 min period to dissolve the precipitated Cu₂O and to reduce the arsenomolybdate. Dilute the contents of each tube to 10 ml with H₂O. Read the absorbance at 540 nm and calculate the μmoles of reducing equivalents in tubes 1, 1', 2, and 2' from your glucose standard curve.

Determination of Inorganic Phosphate. Using the modified Fiske-Subbarow method, assay the following for inorganic phosphate: tubes 3 and 3' (no hydrolysis),

tubes 4 and 4' (7-min hydrolysis), tubes 5 and 5' (total hydrolysis), five standards (0.1, 0.3, 0.5, 0.7, and 0.9 μmoles of phosphate), and a blank. Add 1 ml of acid molybdate reagent and 1 ml of reducing reagent to all tubes, and make up to 10 ml with H_2O. Allow them to stand for 20 min, then read the absorbance of each at 660 nm against the blank. Determine the μmoles of inorganic phosphate in tubes 3, 3', 4, 4', 5, and 5' from your standard curve.

Chromatographic Characterization

Prepare two identical chromatograms (Whatman No. 1 paper square, 20 cm on a side), which carry the following spots, 5–7 mm in diameter: the hydrolyzed material (from tube 6); 1% authentic glucose-1-phosphate; 1% authentic glucose-6-phosphate; 1% isolated, suspected glucose-1-phosphate; 1% glucose; and 1% inorganic phosphate. Develop each chromatogram in ascending fashion, using freshly mixed solvent, which is prepared from 80 ml absolute methanol, 15 ml 88% formic acid, and 5 ml H_2O. (If time permits, you can improve the separation by suspending the chromatogram in the chromatography jar with a fine wire for 2 hrs before allowing the paper to dip into the solvent.) When the chromatograms have developed, dry them, and then spray one with the p-anisidine spray to detect sugars and subject the other to one of the organic phosphate detection procedures listed in following paragraphs.

p-**Anisidine Spray.** Spray the chromatogram with the 1% p-anisidine-HCl spray, then heat the chromatogram at 100–130° C for 3–10 min. Sugars will appear as brown spots. (Note: Acid-hydrolyzed glucose-1-phosphate may contain some hydroxymethylfurfural, which appears as a second, faster-migrating spot.)

Phosphate Detection Systems. Either the modified Hanes-Ischerwood procedure or the iron-sulfosalicylic acid complex methods presented here may be used to detect organic phosphate compounds on chromatograms.

1. Modified Hanes-Ischerwood Spray Procedure. Spray the chromatogram lightly with freshly prepared modified Hanes-Isherwood reagent (25 ml 4% $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, 10 ml 1 N HCl, 5 ml 70% perchloric acid, 60 ml H_2O). Dry the paper in a 100° C oven for a few minutes, but do not allow the paper to darken. The paper will become fragile, so handle it very carefully. Irradiate the paper with a UV lamp held at a distance of 10 cm for 1–10 min. Phosphate containing compounds will appear as blue spots.

2. Iron-Sulfosalicylic Acid Complex Assay of Phosphates. Dip the chromatogram paper containing greater than 0.05 μmoles organic phosphate/spot in a bath of acid-$FeCl_3$ in acetone and immediately hang it up in a hood to dry. When dry, dip the paper in 1.25% sulfosalicylic acid in acetone and again immediately hang it up in a hood to dry. Organic phosphates appear on the dry chromatogram as white spots in a red-brown field. (see Runeckles and Krotkov, 1957).

Report of Results. Determine the R_f values for the various standard and experimental spots on both chromatograms and record them in a table.

Prepare a table similar to Table 16-1. From the weight of isolated glucose-1-phosphate dissolved in H_2O for analysis, calculate the theoretical values for reducing equivalents and inorganic phosphate in each sample assuming a MW of 372 and 100% purity. Then determine the observed values from your analyses. In all cases, report the values as μmoles/0.1 ml of the original glucose-1-phosphate solution analyzed.

TABLE 16-1
Analysis of glucose-1-phosphate isolated from potato phosphorylase reaction

Sample	μmoles of reducing equivalents/0.1 ml		μmoles of inorganic phosphate/0.1 ml	
	Theory	Observed	Theory	Observed
Initial sample (μg/0.1 ml)				
7-min hydrolyzed sample				
Total hydrolysis sample				

How many μmoles of inorganic phosphate contaminate your sample? How many moles of glucose-6-phosphate contaminate your sample? (You have *two* independent determinations of this value. What are they? What do you assume in using them as assays for glucose-6-phosphate? Do they agree? If not, suggest why not.) Do your chromatographic results agree with the conclusions drawn from the chemical analyses?

Determine the extent of hydration of your isolated compound by performing the following operations:

1. Calculate the percentage of 7-min phosphorus (not phosphate) in your sample (e.g., μg P/100 μg sample), assuming that the sample is pure.

2. Calculate the expected percentage of 7-min phosphorus from dipotassium glucose-1-phosphate.

3. Calculate the expected percentage of 7-min phosphorus from dipotassium glucose-1-phosphate dihydrate.

4. Compare your value for the percentage of phosphorus (1) with the two theoretical values (2 and 3), and decide which formula best fits your data. Comment on the reliability of your conclusion.

Weigh and label the remaining glucose-1-phosphate, and turn it in to the instructor.

EXERCISES

1. Describe chemical tests for determining whether a given pure sample of unknown is (a) glucose-6-phosphate, (b) glucose-1-phosphate, (c) β-methylglucoside, (d) glucose, (e) fructose-1,6-diphosphate, or (f) sorbitol.

2. Which of the following compounds yield inorganic phosphate upon 7 min hydrolysis: acetyl phosphate, 3-phosphoglyceraldehyde, ribose-5-phosphate, adenosine-5'-phosphate, ribose-1-phosphate, and pyrophosphate?

3. Point out the chemical similarities between Nelson's test and the Fiske-Subbarow test for phosphate, and between these tests and the Folin-Ciocalteu (Lowry) protein determination.

REFERENCES

Bandurski, R. S., and B. Axelrod. 1951. The Chromatographic Identification of some Biologically Important Phosphate Esters. *J. Biol. Chem., 193:*405.

Cori. C. F., S. P. Colowick. and G. T. Cori. 1937. The Isolation and Synthesis of Glucose-1-Phosphoric Acid. *J. Biol. Chem. 121:*465.

Hanes, C. S., and F. A. Isherwood. 1949. Separation of the Phosphoric Esters. *Nature 164:*1107.

Nelson, N. 1944. A Photometric Adaptation of the Somogyi Method for the Determination of Glucose. *J. Biol. Chem., 153:*375.

Runeckles, V. C. and G. Krotkov. 1957. The Separation of Phosphate Esters and Other Metabolites. *Arch. Biochem. Biophys. 70:*442.