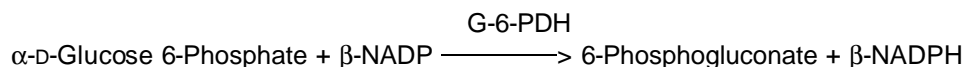
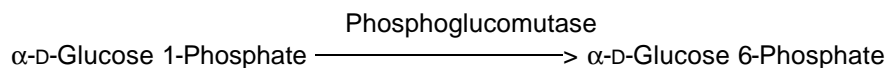
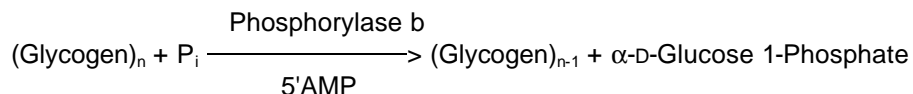
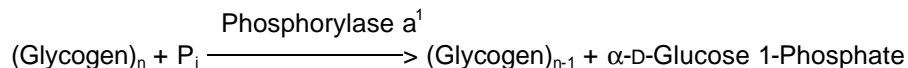


Enzymatic Assay of PHOSPHORYLASE b (EC 2.4.1.1)

PRINCIPLE:



Abbreviations used:

5'-AMP = Adenosine 5'-Monophosphate

P_i = Inorganic Phosphate

PGLUM = Phosphoglucomutase

β-NADP = β-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form

G-6-PDH = Glucose-6-Phosphate Dehydrogenase

β-NADPH = β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form

CONDITIONS: T = 30°C, pH = 6.8, A_{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

- A. 500 mM Potassium Phosphate Buffer, pH 6.8 at 30°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 6.8 at 30°C with 1 M KOH.)
- B. 4% (w/v) Glycogen Solution (Glycogen)
(Prepare 10 ml in deionized water using Glycogen Type III, Sigma Prod. No. G-8876. This may require stirring and heat to solubilize.)
- C. 300 mM Magnesium Chloride Solution (MgCl₂)
(Prepare 5 ml in deionized water using Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250.)

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REAGENTS: (continued)

- D. 100 mM Ethylenediaminetetraacetic Acid Solution (EDTA)
(Prepare 2 ml in deionized water using Ethylenediaminetetraacetic Acid, Tetrasodium Salt, Sigma Stock No. ED4SS.)
- E. 6.5 mM β -Nicotinamide Adenine Dinucleotide Phosphate Solution (β -NADP)
(Prepare 15 ml in deionized water using β -Nicotinamide Adenine Dinucleotide Phosphate, Sodium Salt, Sigma Prod. No. N-0505. **PREPARE FRESH.**)
- F. 0.1% (w/v) α -D-Glucose 1,6-Diphosphate Solution² (G 1,6-DiP)
(Prepare 1 ml in deionized water using α -D-Glucose 1,6-Diphosphate, Cyclohexylammonium Salt, Hydrate, Sigma Prod. No. G-5875.)²
- G. Glucose-6-Phosphate Dehydrogenase Solution (G-6-PDH)
(Immediately before use, prepare a solution containing 10 units/ml in cold deionized water using Glucose-6-Phosphate Dehydrogenase, Sigma Prod. No. G-6378.)
- H. Phosphoglucomutase Solution (PGLUM)
(Immediately before use, prepare a solution containing 10 units/ml in cold deionized water using Phosphoglucomutase, Sigma Prod. No. P-3397.)
- I. 40 mM β -Glycerophosphate with 80 mM Cysteine, pH 6.8 at 30°C (Enzyme Diluent)
(Prepare 50 ml in deionized water using β -Glycerophosphate Disodium Salt, Hydrate, Sigma Prod. No. G-6251 and L-Cysteine, Hydrochloride, Monohydrate, Sigma Prod. No. C-7880. Adjust to pH 6.8 with 1 M NaOH.)
- J. Phosphorylase b Enzyme Solution (Phosphor b)
(Immediately before Step 1: Prepare a solution containing 5 - 10 mg solid/ml of Phosphorylase b in cold Reagent I.)
- K. Diluted Phosphorylase b Enzyme Solution (Dil Phosphor b)
(Immediately before Step 2, prepare a solution containing 0.20 - 0.40 unit/ml of Phosphorylase B in cold Reagent I.)
- L. 100 mM Adenosine 5'-Monophosphate Solution (5'-AMP)³
(Prepare 1 ml in deionized water using Adenosine 5'-Monophosphate, Sodium Salt, Sigma Prod. No. A-1752. **PREPARE FRESH.**)

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PROCEDURE:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into a suitable container:

| | |
|--------------------------------|-------|
| Deionized water | 99.50 |
| Reagent A (Buffer) | 15.00 |
| Reagent B (Glycogen) | 7.50 |
| Reagent C (MgCl ₂) | 0.67 |
| Reagent D (EDTA) | 0.15 |
| Reagent E (β-NADP) | 10.00 |
| Reagent F (G 1,6-DiP) | 0.50 |

Mix by stirring and adjust to pH 6.8 at 30°C with 100 mM HCl or 100 mM NaOH, if necessary.

Step 1:

Pipette (in milliliters) the following reagents into suitable cuvettes:

| | <u>Test</u> | <u>Blank</u> |
|---------------------|-------------|--------------|
| Reaction Cocktail | 2.70 | 2.70 |
| Reagent G (G-6-PDH) | 0.10 | 0.10 |
| Reagent H (PGLUM) | 0.10 | 0.10 |

Mix by inversion and equilibrate to 30°C. Monitor the $A_{340\text{nm}}$ until constant, using a suitably thermostatted spectrophotometer. Then add:

| | | |
|----------------------------|-------|-------|
| Reagent J (Phosphor b) | 0.10 | ----- |
| Reagent I (Enzyme Diluent) | ----- | 0.10 |

Mix by inversion and record the increase in $A_{340\text{nm}}$ for approximately 10 minutes. Obtain the $\Delta A_{340\text{nm}}/\text{minute}$ using the maximum linear rate for both the Test and Blank.⁴

Step 2:

Pipette (in milliliters) the following reagents into suitable cuvettes:

| | | |
|---------------------|-------|-------|
| Reaction Cocktail | 2.70 | 2.70 |
| Reagent G (G-6-PDH) | 0.10 | 0.10 |
| Reagent H (PGLUM) | 0.10 | 0.10 |
| Reagent K (5'-AMP) | 0.050 | 0.050 |

Enzymatic Assay of PHOSPHORYLASE b (EC 2.4.1.1)

PROCEDURE: (continued)

Mix by inversion and equilibrate to 30°C. Monitor the $A_{340\text{nm}}$ until constant, using a suitably thermostatted spectrophotometer. Then add:

| | <u>Test</u> | <u>Blank</u> |
|----------------------------|-------------|--------------|
| Reagent K (Dil Phosphor b) | 0.10 | ----- |
| Reagent I (Enzyme Diluent) | ----- | 0.10 |

Immediately mix by inversion and record the increase in $A_{340\text{nm}}$ for approximately 5 minutes. Obtain the $\Delta A_{340\text{nm}}/\text{minute}$ using the maximum linear rate⁵ for both the Test and Blank.

CALCULATIONS:

Phosphorylase a units/ml enzyme =

$$\frac{(\Delta A_{340}/\text{min Test without 5'AMP} - \Delta A_{340}/\text{min Blank without 5'AMP})(3.00)(\text{df})}{(6.22)(0.1)}$$

$$\text{Phosphorylase a units/mg solid} = \frac{\text{Phosphorylase a units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

Phosphorylase a and b units/ml enzyme =

$$\frac{(\Delta A_{340}/\text{min Test with 5'AMP} - \Delta A_{340}/\text{min Blank with 5'AMP})(3.05)(\text{df})}{(6.22)(0.1)}$$

$$\text{Phosphorylase a and b units/mg solid} = \frac{\text{Phosphorylase a and b units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

Phosphorylase b units/mg solid = Phosphorylase a and b units/mg solid - Phosphorylase a units/mg solid

3.00 = Total volume (in milliliters) of Phosphorylase A assay

3.05 = Total volume (in milliliters) of Phosphorylase A and B assay

df = Dilution factor

6.22 = Millimolar extinction coefficient of β -NADPH at 340 nm

0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

Enzymatic Assay of PHOSPHORYLASE b (EC 2.4.1.1)

UNIT DEFINITION:

One unit will form 1.0 μ mole of α -D-glucose 1-phosphate from glycogen and orthophosphate in the presence of adenosine 5'-monophosphate per minute at pH 6.8 at 30°C, measured in a system containing phosphoglucomutase, β -nicotinamide adenine dinucleotide phosphate, and glucose-6-phosphate dehydrogenase.

FINAL ASSAY CONCENTRATION:

In a 3.05 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 0.2% (w/v) glycogen, 1.3 mM magnesium chloride, 0.10 mM ethylenediaminetetraacetic acid, 0.43 mM β -nicotinamide adenine dinucleotide phosphate, 0.0003% (w/v) α -D-glucose 1,6-diphosphate, 1 unit glucose-6-phosphate dehydrogenase, 1 unit phosphoglucomutase, 1.6 mM adenosine 5'-monophosphate, and 0.02 - 0.04 unit phosphorylase b.

REFERENCES:

Bergmeyer, H.U., Gawehn, K., and Grassl, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.U. ed.) 2nd ed., Volume I, 505-507, Academic Press, Inc., New York, NY

Fischer, E.H. and Krebs, E.G. (1962) *Methods in Enzymology*, Volume V, 369-373

NOTES:

1. Phosphorylase a activity is present in the phosphorylase b preparation and must be accounted for in the enzyme assay.
2. α -D-Glucose 1,6-Diphosphate is an activator of phosphoglucomutase.
3. Phosphorylase b is enzymatically inactive in the absence of 5'-AMP.
4. The maximal rate is reached within approximately 3 minutes. This maximal rate is due to any phosphorylase a activity which may be present.
5. The maximum linear rate should not exceed a $\Delta A_{340\text{nm}}$ of 0.1 absorbance unit/minute.

**Enzymatic Assay of PHOSPHORYLASE b
(EC 2.4.1.1)**

NOTES: (continued)

6. Glucose-6-Phosphate Dehydrogenase Unit Definition: One unit will oxidize 1.0 μ mole of D-glucose 6-phosphate to 6-phospho-D-gluconate per minute in the presence of β -NADP at pH 7.4 at 25°C.
7. Phosphoglucomutase Unit Definition: One unit will convert 1.0 μ mole of α -D-glucose 1-phosphate to α -D-glucose 6-phosphate per minute at pH 7.4 at 30°C.
8. This assay is based on the cited references.
9. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

This procedure is for informational purposes. For a current copy of Sigma's quality control procedure contact our Technical Service Department.