

Enzymatic Assay of PYRUVATE DEHYDROGENASE

PRINCIPLE:

Pyruvate + P_i + mPMS (oxidized) $\xrightarrow[\text{TPP, FAD}]{\text{Pyruvate Dehydrogenase}}$ Acetyl Phosphate + CO₂ + mPMS

2 mPMS (reduced) + NTB \longrightarrow 2 mPMS (oxidized) + Diformazan

Abbreviations used:

P_i = Inorganic Phosphate

mPMS = 1-Methoxy-5-Methylphenazinium Methyl Sulfate

TPP = Thiamine Pyrophosphate

NBT = Nitro Blue Tetrazolium

FAD = Flavin Adenine Dinucleotide

CONDITIONS: T = 37°C, pH = 6.3, A_{570nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

- A. 150 mM Potassium Phosphate Buffer with 1.5% (w/v) Triton¹ X-100, pH 6.3 at 37°C (Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379, and Triton¹ X-100, Sigma Stock No. X-100. Adjust to pH 6.3 at 37°C with 1 M HCl or 1 M KOH.)
- B. 300 mM Pyruvate Solution (Prepare 10 ml in deionized water using Pyruvic Acid, Sodium Salt, Sigma Prod. No. P-2256. **PREPARE FRESH.**)
- C. 0.15 mM Flavin Adenine Dinucleotide Solution (FAD) (Prepare 20 ml in deionized water using Flavin Adenine Dinucleotide, Disodium Salt, Sigma Prod. No. F-6625. **PREPARE FRESH.**)

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REAGENTS: (continued)

- D. 3.0 mM Cocarboxylase (Thiamine Pyrophosphate) Solution (TPP)
(Prepare 5 ml in deionized water using Cocarboxylase, Sigma Prod. No. C-8754. **PREPARE FRESH.**)
- E. 7.5 mM Ethylenediaminetetraacetic Acid Solution (EDTA)
(Prepare 10 ml in deionized water using Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate, Sigma Stock No. ED2SS.)
- F. 75 mM Magnesium Sulfate Solution ($MgSO_4$)
(Prepare 10 ml in deionized water using Magnesium Sulfate, Heptahydrate, Sigma Prod. No. M-1880.)
- G. 0.5% (w/v) Triton¹ X-100 Solution
(Prepare 25 ml in deionized water using Triton¹ X-100, Sigma Stock No. X-100.)
- H. 1.0 mM 1-Methoxy-5-Methylphenazinium Methyl Sulfate with 10 mM Nitro Blue Tetrazolium Solution (mPMS/NBT)
(Prepare 10 ml in cold Reagent G using 1-Methoxy-5-Methylphenazinium Methyl Sulfate, Sigma Prod. No. M-8640, and Nitro Blue Tetrazolium, Sigma Prod. No. N-6876. Store in an amber bottle. **PREPARE FRESH.**)
- I. 50 mM Potassium Phosphate Buffer, pH 6.3 at 37°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 6.3 at 37°C with 1 M HCl or 1 M KOH.)
- J. Pyruvate Dehydrogenase Enzyme Solution
(Immediately before use, prepare a solution containing 0.1 - 0.5 unit/ml of Pyruvate Dehydrogenase in cold Reagent I.)

PROCEDURE:

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

	<u>Test</u>	<u>Blank</u>
Reagent A (Buffer)	1.00	1.00
Reagent B (Pyruvate)	0.50	0.50
Reagent C (FAD)	0.20	0.20
Reagent D (TPP)	0.20	0.20
Reagent E (EDTA)	0.40	0.40
Reagent F ($MgSO_4$)	0.40	0.40
Reagent H (mPMS/NBT)	0.30	0.30

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PROCEDURE: (continued)

Mix and equilibrate to 37°C. Monitor the $A_{570\text{nm}}$ until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent I (Diluent)	-----	0.10
Reagent J (Enzyme Solution)	0.10	-----

Immediately mix by inversion and record the increase in $A_{570\text{nm}}$ for approximately 5 minutes. Obtain the $r A_{570\text{nm}}/\text{minute}$ using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(r A_{570\text{nm}}/\text{min Test} - r A_{570\text{nm}}/\text{min Blank})(2)(3.1)(\text{df})}{(43.8)(0.1)}$$

2 = One mole of Diformazan produced per 2 moles of reduced mPMS

3.1 = Total volume (in milliliters) of assay

df = Dilution factor

43.8 = Millimolar extinction coefficient of Diformazan at 570 nm

0.1 = Volume (in milliliters) of enzyme used

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

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

UNIT DEFINITION:

One unit will produce 1.0 μmole of acetyl phosphate and CO_2 from pyruvate and inorganic phosphate per minute at pH 6.3 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 3.10 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 0.05% (v/v) Triton X-100, 48 mM pyruvate, 0.0097 mM FAD, 0.19 mM TPP, 0.97 mM EDTA, 9.7 mM magnesium sulfate, 0.097 mM 1-methoxy-5-methylphenazinium methyl sulfate, 0.97 mM nitro blue tetrazolium, 0.01 - 0.05 unit pyruvate dehydrogenase.

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REFERENCE:

Fine, I.H. and Costello, L.A. (1963) *Methods in Enzymology*, VI, 958-972.

NOTES:

1. Triton is a registered trademark of Union Carbide Chemicals and Plastics Co., Inc.
2. This assay is based on the cited reference.
3. 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.