Enzymatic Assay of PYRUVATE DEHYDROGENASE

PRINCIPLE:

\[
\text{Pyruvate} + P_i + \text{mPMS (oxidized)} \xrightarrow{\text{Pyruvate Dehydrogenase} \quad \text{TPP, FAD}} \text{Acetyl Phosphate} + \text{CO}_2 + \text{mPMS}
\]

\[
2 \text{mPMS (reduced)} + \text{NTB} \quad \rightarrow \quad 2 \text{mPMS (oxidized)} + \text{Diformazan}
\]

Abbreviations used:
- P\textsubscript{i} = Inorganic Phosphate
- mPMS = 1-Methoxy-5-Methylphenazinium Methyl Sulfate
- TPP = Thiamine Pyrophosphate
- NBT = Nitro Blue Tetrazolium
- FAD = Flavin Adenine Dinucleotide

CONDITIONS:  \( T = 37^\circ C, \ \text{pH} = 6.3, \ A_{570\text{nm}}, \ \text{Light path} = 1 \ cm \)

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 150 mM Potassium Phosphate Buffer with 1.5% (w/v) Triton\textsuperscript{1} X-100, pH 6.3 at 37°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, and Triton\textsuperscript{1} 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.  \textbf{PREPARE FRESH}.)

C. 0.15 mM Flavin Adenine Dinucleotide Solution (FAD)
(Prepare 20 ml in deionized water using Flavin Adenine Dinucleotide, Disodium Salt.  \textbf{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, **PREPARE FRESH**.)

E. 7.5 mM Ethylenediaminetetraacetic Acid Solution (EDTA)  
(Prepare 10 ml in deionized water using Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate.)

F. 75 mM Magnesium Sulfate Solution (MgSO$_4$)  
(Prepare 10 ml in deionized water using Magnesium Sulfate, Heptahydrate.)

G. 0.5% (w/v) Triton$^1$ X-100 Solution  
(Prepare 25 ml in deionized water using Triton$^1$ 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, and Nitro Blue Tetrazolium,  
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.  
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:**

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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A (Buffer)</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Reagent B (Pyruvate)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent C (FAD)</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Reagent D (TPP)</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Reagent E (EDTA)</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Reagent F (MgSO$_4$)</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Reagent H (mPMS/NBT)</td>
<td>0.30</td>
<td>0.30</td>
</tr>
</tbody>
</table>
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PROCEDURE: (continued)

Mix and equilibrate to 37°C. Monitor the A\textsubscript{570nm} 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\textsubscript{570nm} for approximately 5 minutes. Obtain the r A\textsubscript{570nm}/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

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

2 = One mole of Diformazan produced per 2 moles of reduced mPMS
3.1 = Total volume (in milliliters) of assay
\text{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\textsubscript{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:


NOTES:

1. Triton is a registered trademark of Union Carbide Chemicals and Plastics Co., Inc.

2. This assay is based on the cited reference.