Enzymatic Assay of PYRUVATE CARBOXYLASE  
(EC 6.4.1.1)

PRINCIPLE:

\[
\text{PC}
\]

\[
\text{Pyruvate + ATP + HCO}_3^- \rightarrow \text{Oxalacetate + ADP + P}_i
\]

\[
\text{AcCoA}
\]

\[
\text{MDH}
\]

\[
\text{Oxalacetate + } \beta\text{-NADH} \rightarrow \text{Malate + } \beta\text{-NAD}
\]

Abbreviations used:
ATP = Adenosine 5'-Triphosphate
PC = Pyruvate Carboxylase
AcCoA = Acetyl Coenzyme A
ADP = Adenosine 5'-Diphosphate
P\textsubscript{i} = Inorganic Phosphate
\beta\text{-NADH} = \beta\text{-Nicotinamide Adenine Dinucleotide, Reduced Form}
MDH = Malic Dehydrogenase
\beta\text{-NAD} = \beta\text{-Nicotinamide Adenine Dinucleotide, Oxidized Form}

CONDITIONS: T = 30°C, pH = 7.8, A\textsubscript{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 135 mM Triethanolamine Buffer with 7 mM Magnesium Sulfate, 9 mM Pyruvic Acid, and 0.15% (w/v) Bovine Serum Albumin, pH 8.0 at 30°C (Substrate)  
(Prepare 100 ml in deionized water using Triethanolamine Hydrochloride, Magnesium Sulfate, Anhydrous, Pyruvic Acid, Sodium Salt, and Albumin, Bovine. Adjust to pH 8.0 at 30°C with 1 M HCl.)

B. 0.3 mM Acetyl Coenzyme A Solution  
(Prepare 10 ml in deionized water using Acetyl Coenzyme A, Sodium Salt.  
PREPARE FRESH.)
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REAGENTS: (continued)

C. Malic Dehydrogenase Enzyme Solution (AcCoA/MDH)  
(Immediately before use, add 150 units of Malic Dehydrogenase, to 5 ml of Reagent B. Bring the solution to a total volume of 10.0 ml with deionized water.)

D. 100 mM Triethanolamine Buffer with 30 mM Adenosine 5'-Triphosphate and 450 mM Potassium Bicarbonate, pH 8.0 at 30°C (ATP/KHCO₃)  
(Prepare 10 ml in deionized water using Triethanolamine Hydrochloride, Adenosine 5'-Triphosphate, Disodium Salt, and Potassium Bicarbonate. Adjust to pH 8.0 at 30°C with 1 M KOH.)

E. 2.6 mM β-Nicotinamide Adenine Dinucleotide, Reduced Form (β-NADH)  
(Prepare 10 ml in deionized water using β-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt.)

F. 50 mM Tris HCl Buffer with 50% (v/v) Glycerol, 2 mM Magnesium Acetate, and 1 mM Ethylenediaminetetraacetic Acid, pH 7.4 at 30°C (Enz Dil)  
(Prepare 50 ml in deionized water using Trizma Base, Glycerol, Magnesium Acetate, Tetrahydrate, and Ethylenediaminetetraacetic Acid, Tetrasodium Salt, Hydrate. Adjust to pH 7.4 at 30°C with 1 M HCl.)

G. Pyruvate Carboxylase Enzyme Solution (PC)  
(Immediately before use, prepare a solution containing approximately 30 - 90 units/ml of Pyruvate Carboxylase in cold Reagent F.)

PROCEDURE:

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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Substrate)</td>
<td>20.00</td>
</tr>
<tr>
<td>C (AcCoA/MDH)</td>
<td>5.00</td>
</tr>
<tr>
<td>E (β-NADH)</td>
<td>2.50</td>
</tr>
</tbody>
</table>

Mix by swirling. Adjust to pH 7.8 at 30°C if necessary, with either 1 M HCl or 1 M KOH.
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PROCEDURE:

Pipette (in milliliters) the following reagents into a suitable container:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Cocktail</td>
<td>2.90</td>
<td>2.90</td>
</tr>
<tr>
<td>Reagent G (PC)</td>
<td>0.005</td>
<td>------</td>
</tr>
<tr>
<td>Reagent F (Enz Dil)</td>
<td>------</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 30°C. Monitor the A$_{340\text{nm}}$ until constant using a suitably thermostatted spectrophotometer. Record the $\Delta$A$_{340\text{nm}}$/minute of the Test.$^1$ Then add:

| Reagent D (ATP/KHCO$_3$) | 0.10  | 0.10  |

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

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340\text{nm}}/\text{min Test} - \Delta A_{340\text{nm}}/\text{min Blank})(3.005)(\text{df})}{(6.22)(0.005)}$$

3.005 = Total volume (in milliliters) of the assay  
$\text{df}$ = Dilution factor  
6.22 = Millimolar extinction coefficient of β-NADH at 340 nm  
0.005 = Volume (in milliliter) of pyruvate carboxylase used in the assay

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

UNIT DEFINITION:

One unit will convert 1.0 µmole of pyruvate and CO$_2$ to oxalacetate per minute at pH 7.8 at 30°C.
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FINAL CONCENTRATIONS:

In a 3.005 ml reaction mix, the final concentrations are 134 mM triethanolamine, 5 mM magnesium sulfate, 7 mM pyruvic acid, 0.12% (w/v) bovine serum albumin, 0.23 mM β-nicotinamide adenine dinucleotide, reduced form, 0.05 mM acetyl coenzyme A, 2.63 units malic dehydrogenase, 1 mM adenosine 5’-triphosphate, 15 mM potassium bicarbonate, 0.05% (v/v) glycerol, 0.002 mM magnesium acetate, 0.001 mM ethylenediaminetetraacetic acid, 0.05 mM Tris, 0.15 - 0.45 unit pyruvate carboxylase.

REFERENCE:


NOTES:

1. Lactic Dehydrogenase is the principle contaminant that may interfere with the assay for pyruvate carboxylase. If the ΔA₃₄₀nm/min is not zero, it must be subtracted from the ΔA₃₄₀nm/min for the Test after the addition of Reagent D (ATP/KHCO₃).

2. Malic Dehydrogenase Unit Definition: One unit will convert 1.0 μmole of oxalacetate and β-NADH to L-malate and β-NAD per minute at pH 7.5 at 25°C.

3. This assay is based on the cited reference.