Enzymatic Assay of ß-NAD SYNTHETASE
(EC 6.3.5.1)

PRINCIPLE:
ATP + deamido-NAD\(^+\) + NH\(_3\)  \(\beta\)-NAD Synthetase\(^1\)  \(\text{Mg}^{2+}, \text{K}^+\) > AMP + NAD\(^+\) + ppi

Abbreviations used:
ATP = Adenosine 5’-Triphosphate
Deamido-NAD\(^+\) = Nicotinic Acid Adenine Dinucleotide
AMP = Adenosine 5’-Monophosphate
ß-NAD = ß-Nicotinamide Adenine Dinucleotide, Oxidized Form
ppi = Inorganic Pyrophosphate

CONDITIONS:  \(T = 37^\circ\text{C}, \ pH = 8.5, A_{340\text{nm}}, \ \text{Light path} = 1 \ \text{cm}\)

METHOD:  Spectrophotometric Stop Rate Determination

REAGENTS:

A.  120 mM Tris HCl Buffer, pH 8.5 at 37°C
(Prepare 100 ml in deionized water using Trizma Base,
Adjust to pH 8.5 at 37°C with
1 M HCl.)

B.  30 mM Nicotinic Acid Adenine Dinucleotide Solution
(deamido-NAD\(^+\))
(Prepare 1 ml in deionized water using Nicotinic Acid
Adenine Dinucleotide.)

C.  60 mM Adenosine 5’-Triphosphate Solution (ATP)
(Prepare 1 ml in deionized water using Adenosine
5’-Triphosphate, Disodium Salt.)

D.  30 mM Ammonium Sulfate Solution \(((\text{NH}_4)_2\text{SO}_4)\)
(Prepare 1 ml in deionized water using Ammonium
Sulfate.)

E.  1% \((\text{w/v})\) Bovine Serum Albumin Solution (BSA)
(Prepare 1 ml in deionized water using Albumin,
Bovine.)
Enzymatic Assay of \( \beta \)-NAD SYNTHETASE
(EC 6.3.5.1)

REAGENTS: (continued)

F. 240 mM Magnesium Chloride Solution (\( \text{MgCl}_2 \))
(Prepare 10 ml in deionized water using Magnesium Chloride, Anhydrous.)

G. 120 mM Potassium Chloride Solution (\( \text{KCl} \))
(Prepare 10 ml in deionized water using Potassium Chloride.)

H. Alcohol Dehydrogenase Enzyme Solution (ADH)
(Immediately before use, prepare a solution containing 200 units/ml of Alcohol Dehydrogenase, in cold deionized water.)

I. 95\% (v/v) Ethanol (EtOH)
(Prepare 1 ml in deionized water using 200 Proof Ethyl Alcohol, available from Quantum Chemical Company.)

J. \( \beta \)-NAD Synthetase Enzyme Solution (\( \beta \)-NAD Synth)
(Immediately before use, prepare a solution containing 0.15 - 1.0 unit/ml of \( \beta \)-NAD Synthetase in cold deionized water.)

PROCEDURE:

Pipette (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>1.55</td>
<td>1.55</td>
</tr>
<tr>
<td>Reagent B (deamido-NAD(^+))</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent C (ATP)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent D ((NH(_4))(_2)SO(_4))</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent E (BSA)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent F (( \text{MgCl}_2 ))</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Reagent G (( \text{KCl} ))</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent H (ADH)</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Reagent J (( \beta )-NAD Synth)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>
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PROCEDURE:  (continued)

Mix by inversion and equilibrate to 37°C. Monitor the $A_{340\text{nm}}$ until constant, using a suitably thermostatted spectrophotometer. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent I (EtOH)</td>
<td>0.05</td>
<td>------</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>------</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Immediately mix by swirling and incubate at 37°C for exactly 2 minutes. Immediately transfer to a boiling water bath for one minute. Remove the Test and Blank and place on ice until cooled. Filter to clarify using a 0.2 µm filter. Record the $A_{340\text{nm}}$ using a suitable spectrophotometer for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(r A_{340\text{nm}}/\text{min Test} - r A_{340\text{nm}}/\text{min Blank})(3)(df)}{(2)(6.22)(0.05)}$$

3 = Total volume (in milliliters) of assay
df = Dilution factor
2 = Time (in minutes) of assay as per the Unit Definition
6.22 = Millimolar extinction coefficient of β-NADH at 340 nm
0.05 = Volume (in milliliter) of enzyme use

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

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

UNIT DEFINITION:

One unit will form 1.0 µmole of β-NAD from nicotinic acid adenine dinucleotide per minute at pH 8.5 at 37°C.
Enzymatic Assay of β-NAD SYNTHETASE  
(EC 6.3.5.1)

FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 62 mM Tris, 1 mM nicotinic acid adenine dinucleotide, 2 mM adenosine 5'-triphosphate, 1 mM ammonium sulfate, 0.03% (w/v) bovine serum albumin, 20 mM magnesium chloride, 20 mM potassium chloride, 1.6% (v/v) ethanol, 40 units alcohol dehydrogenase, and 0.0075 - 0.05 unit β-NAD synthetase.

REFERENCE:


NOTES:

1. Glutamine can also act as a substrate.

2. The alcohol dehydrogenase used as a coupling enzyme must be suitable for the recycling assay of β-NAD and β-NADH.  (It must contain less than 0.005 mole of β-NAD and β-NADH per mole of ADH.)

3. This assay is based on the cited reference.

4. Alcohol Dehydrogenase Unit Definition: One unit will convert 1.0 µmole of ethanol to acetaldehyde per minute at pH 8.8 at 25°C.