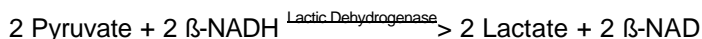
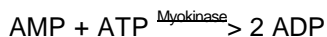
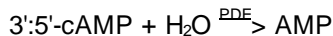


Enzymatic Assay of PHOSPHODIESTERASE, 3':5'-CYCLIC NUCLEOTIDE ACTIVATOR DEFICIENT¹

PRINCIPLE:



Abbreviations used:

3':5'-cAMP = Adenosine 3':5'-Cyclic Monophosphate

PDE = Phosphodiesterase, 3':5'-Cyclic Nucleotide

AMP = Adenosine 5'-Monophosphate

ATP = Adenosine 5'-Triphosphate

ADP = Adenosine 5'-Diphosphate

PEP = Phospho(enol)pyruvate

β -NADH = β -Nicotinamide Adenine Dinucleotide, Reduced Form

β -NAD = β -Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS: T = 30°C, pH = 7.5, $A_{340\text{nm}}$, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

- A. 100 mM Tris HCl Buffer with 80 mM Potassium Chloride and 9.7 mM Magnesium Sulfate, pH 7.5 at 30°C
(Prepare 50 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503, Potassium Chloride, Sigma Prod. No. P-4504, and Magnesium Sulfate, Heptahydrate, Sigma Prod. No. M-1880. Adjust to pH 7.5 at 30°C with 1 M NaOH.)
- B. 0.9 mM Phospho(enol)pyruvate Solution (PEP)
(Prepare 50 ml in Reagent A using Phospho(enol)pyruvate, Monopotassium Salt, Sigma Prod. No. P-7127. **PREPARE FRESH.**)

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REAGENTS: (continued)

- C. 30 mM Adenosine 5'-Triphosphate Solution (ATP)
(Prepare 2 ml in deionized water using Adenosine 5'-Triphosphate, Disodium Salt, Sigma Prod. No. A-5394. Adjust to pH 7.5 at 30°C with Sodium Bicarbonate, Sigma Prod. No. S-8875. **PREPARE FRESH.**)
- D. 120 mM Adenosine 3':5'-Cyclic Monophosphate Solution (3':5'-cAMP)
(Prepare 2 ml in deionized water using Adenosine 3':5'-Cyclic Monophosphate, Sodium Salt, Sigma Prod. No. A-6885. **PREPARE FRESH.**)
- E. PK/LDH Enzymes Suspension²
(Use PK/LDH Enzymes Suspension, Sigma Stock No. 40-7.)
- F. Myokinase Enzyme Solution (MK)
(Immediately before use, prepare a solution containing 200 units/ml in cold deionized water using Myokinase, Sigma Prod. No. M-3003.)
- G. 1.75 mM Calcium Acetate Solution (Ca(OAc)₂)
(Prepare 10 ml in deionized water using Calcium Acetate, Sigma Prod. No. C-1000.)
- H. 6.4 mM β-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (β-NADH)
(Dissolve the contents of one 5 mg vial of β-Nicotinamide Adenosine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-105, in the appropriate volume of deionized water. **PREPARE FRESH.**)
- I. Phosphodiesterase, 3':5'-Cyclic Nucleotide Activator Solution (Calmodulin)
(Immediately before use, prepare a solution containing 300 units/ml in deionized water using Phosphodiesterase, 3':5'-Cyclic Nucleotide Activator, Sigma Prod. No. P-2277.)
- J. Phosphodiesterase, 3':5'-Cyclic Nucleotide Enzyme, Activator Deficient Solution
(Immediately before use, prepare a solution containing 0.2 unit/ml of Phosphodiesterase, 3':5'-Cyclic Nucleotide, Activator Deficient, in cold deionized water.)

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PROCEDURE:

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

Deionized Water	6.45
Reagent B (PEP)	19.95
Reagent C (ATP)	0.30
Reagent D (3':5'-cAMP)	0.30
Reagent E (PK/LDH)	0.15
Reagent F (MK)	0.30
Reagent G (Ca(OAc) ₂)	0.45
Reagent H (β-NADH)	0.60

Mix by swirling and adjust to pH 7.5 at 30°C with 1 M HCl or 1 M NaOH, if necessary.

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

	Non-Activated (NA)		Activated (A)			
	Test-NA	Blank-NA	Test-A	Blank-A		
Deionized Water	0.10	0.20			-----	0.10
Reaction Cocktail	3.00	3.00			3.00	3.00
Reagent I (Calmodulin)	-----	-----	0.10	0.10		

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

Reagent J (Enzyme Solution)	0.10	-----	0.10	-----
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Immediately mix by inversion and record the decrease in A_{340nm} for approximately 15 minutes. Obtain the $\Delta A_{340nm}/\text{minute}$ using the maximum linear rate for both the Test and Blank of the activated and non-activated systems.

**Enzymatic Assay of PHOSPHODIESTERASE, 3':5'-CYCLIC NUCLEOTIDE
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CALCULATIONS:

Non-Activated Enzyme:

Units/mg enzyme =

$$\frac{(\bar{r} A_{340\text{nm}}/\text{min Test-NA}) - (\bar{r} A_{340\text{nm}}/\text{min Blank-NA})(3.2)(\text{df})}{(2)(6.22)(0.1)}$$

Activated Enzyme:

Units/mg enzyme =

$$\frac{(\bar{r} A_{340\text{nm}}/\text{min Test-A}) - (\bar{r} A_{340\text{nm}}/\text{min Blank-A})(3.2)(\text{df})}{(2)(6.22)(0.1)}$$

3.2 = Total volume (in milliliters) of assay

df = Dilution factor

2 = 2 μ moles of β -NAD produced per μ mole of 3':5'-cAMP hydrolyzed

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

0.1 = Volume (in milliliters) of enzyme used in the assay

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

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

UNIT DEFINITION:

One unit will hydrolyze 1.0 μ mole of 3':5'-cyclic-AMP to 5'-AMP per minute at pH 7.5 at 30°C.

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FINAL ASSAY CONCENTRATIONS:

In a 3.20 ml reaction mix, the final concentrations are 66 mM Tris, 53 mM potassium chloride, 6.4 mM magnesium sulfate, 0.59 mM phospho(enol)pyruvate, 0.30 mM adenosine 5'-triphosphate, 1.2 mM adenosine 3':5'-cyclic monophosphate, 11 units pyruvate kinase, 16 units lactic dehydrogenase, 6.3 units myokinase, 0.026 mM calcium acetate, 0.13 mM β -NADH and 0.02 unit phosphodiesterase, 3':5'-cyclic nucleotide, activator deficient. (In the activated test 30 units of calmodulin are present.)

NOTES:

1. This assay is not to be used for Sigma Prod. No. P-0134.
2. Contains not less than 700 Pyruvate Kinase units and 1000 L-Lactic Dehydrogenase units per ml.
3. L-Lactic Dehydrogenase Unit Definition: One unit will reduce 1.0 μ mole of pyruvate to L-lactate per minute at pH 7.5 at 37°C.
4. Pyruvate Kinase Unit Definition: One unit will convert 1.0 μ mole of phospho(enol)pyruvate to pyruvate per minute at pH 7.6 at 37°C.
5. Myokinase Unit Definition: One unit will convert 2.0 μ moles of ADP to ATP and AMP per minute at pH 7.6 at 37°C.
6. Phosphodiesterase 3':5'-Cyclic Nucleotide Activator Unit Definition: One unit will stimulate 0.016 activated units of phosphodiesterase 3':5'-cyclic nucleotide, P-0520, to 50% of the maximum activity of the enzyme when saturated with activator, in the presence of 0.01 mM Ca^{++} , pH 7.5 at 30°C.
7. All product and stock numbers, unless otherwise indicated, are Sigma product and stock numbers.

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