Determination of the Concentration and Molecular Weight of ADENOSINE 5'-DIPHOSPHATE

PRINCIPLE:

PEP + ADP $\xrightarrow{PK}$ Pyruvate + ATP

Pyruvate + ß-NADH $\xrightarrow{LDH}$ Lactate + ß-NAD

Abbreviations used:
PEP = Phospho(enol)pyruvate
ADP = Adenosine 5'-Diphosphate
PK = Pyruvate Kinase
ATP = Adenosine 5'-Triphosphate
ß-NADH = ß-Nicotinamide Adenine Dinucleotide, Reduced Form
LDH = Lactate Dehydrogenase
ß-NAD = ß-Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS: T = 25°C, pH = 7.6, A_{340nm}, Light path = 1 cm

METHOD: Spectrophotometric Determination

REAGENTS:

A. 150 mM Triethanolamine HCl Buffer, pH 7.6 at 25°C
(Prepare 100 ml in deionized water using Triethanolamine, Hydrochloride, Sigma Prod. No. T-1502. Adjust to pH 7.6 at 25°C with 1 M NaOH.)

B. 32 mM Phospho(enol)pyruvate, 83 mM Magnesium Sulfate, and 135 mM Potassium Chloride Solution (PEP)
(Prepare 2 ml in deionized water using Phospho(enol)pyruvate, Tri(cyclohexylammonium)Salt, Sigma Prod. No. P-7252, Magnesium Sulfate, Sigma Prod. No. M-1880, and Potassium Chloride, Sigma Prod. No. P-4504.)

C. 3.8 mM ß-Nicotinamide Adenine Dinucleotide, Reduced Form (ß-NADH)
(Prepare 2 ml in Reagent A using ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Prod. No. N-8129.)

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REAGENTS: (continued)

D. Adenosine 5'-Diphosphate Solution (ADP)
(Weigh approximately 2.5 mg of Adenosine 5'-Diphosphate and dissolve in 25 ml of deionized water.)

E. L-Lactic Dehydrogenase Enzyme Suspension (LDH)[1]
(Use L-Lactic Dehydrogenase, Sigma Prod. No. L-2500.)

F. Pyruvate Kinase Enzyme Suspension (PK)[2]
(Use Pyruvate Kinase, Sigma Prod. No. P-1506.)

PROCEDURE:

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

	Test	Blank
Reagent A (Buffer)	1.70	1.80
Reagent B (PEP)	0.15	0.15
Reagent C (ß-NADH)	0.10	------
Reagent D (ADP)	1.00	1.00
Reagent E (LDH)	0.01	0.01

Mix by inversion and equilibrate to 25°C using a suitably thermostatted spectrophotometer. Record the initial A_{340nm} for both the Test and Blank. Then add:

Reagent F (PK)	0.02	0.02

Immediately mix by inversion and allow the reaction to proceed to completion (approximately 5 minutes). Record the final A_{340nm} for both the Test and Blank.

CALCULATION:

$\Delta A = A_i - A_f$ $\quad A_i$ = Initial Absorbance, A_f = Final Absorbance

$$\text{Micromoles ADP/weighed sample} = \frac{(\Delta A \text{ Test} - \Delta A \text{ Blank})(2.98)(25)}{6.22}$$

2.98 = Total volume (in milliliters) of assay
25 = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADH at 340 nm

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CALCULATIONS: (continued)

$$\text{Apparent Molecular Weight} = \frac{\text{mg sample weighed} \times 1000}{\mu\text{moles ADP/weighed sample}}$$

FINAL ASSAY CONCENTRATIONS:

In a 2.98 ml reaction mix, the final concentrations are 91 mM triethanolamine, 1.6 mM phospho(enol)pyruvate, 4.2 mM magnesium sulfate, 6.8 mM potassium chloride, 0.13 mM ß-nicotinamide adenine dinucleotide, reduced form, 40 units pyruvate kinase, 100 units lactic dehydrogenase, and varying amounts of adenosine 5'-diphosphate.

REFERENCE:

Grassl, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.U. ed.) 2nd ed., Volume 4, 2149-2152, Academic Press, Inc, New York, NY

NOTES:

1. Contains not less than 10,000 units of L-lactic dehydrogenase per ml.
2. Contains not less than 2,000 units of pyruvate kinase per ml.
3. Lactic Dehydrogenase Unit Definition: One unit will reduce 1.0 µmole 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 µmole of phospho(enol)pyruvate to pyruvate per minute at pH 7.6 at 37°C.
5. This assay is based on the cited reference.
6. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.