Enzymatic Assay of OROTATE REDUCTASE
(EC 1.3.1.14)

PRINCIPLE:

Orotate + ß-NADH $\xrightarrow{\text{Orotate Reductase}}$ Dihydroorotate + ß-NAD

Abbreviations used:
ß-NADH = ß-Nicotinamide Adenine Dinucleotide, Reduced Form
ß-NAD = ß-Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS: T = 25°C, pH = 6.5, A_{340nm}, Light Path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 400 mM Sodium Phosphate Buffer, pH 6.5 at 25°C
(Prepare 200 ml in deionized water using Sodium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. S-0751. Adjust to pH 6.5 at 25°C with 1 M NaOH.)

B. 400 mM Cysteine Solution (Cys)
(Prepare 10 ml in deionized water using L-Cysteine Hydrochloride, Monohydrate, Sigma Prod. No. C-7880. Adjust to pH 6.5 at 25°C with 1 M NaOH.)

C. 10 mM Orotic Acid Solution (Orotic)
(Prepare 10 ml in 10 mM NaOH using Orotic Acid, Monohydrate, Sigma Prod. No. O-2625. Adjust to pH 6.5 with 10 mM HCl.)

D. 1.6 mM ß-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (ß-NADH)
(Prepare 2 ml in Reagent A using ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma
Prod. No. N-8129, or dissolve the contents of one 5 mg vial of ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-105, in the appropriate volume of Reagent A. **PREPARE FRESH**.)

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REAGENTS: (continued)

E. 3 mM Flavin Adenine Dinucleotide Solution (FAD) (Prepare 1 ml in deionized water using Flavin Adenine Dinucleotide, Disodium Salt, Sigma Prod. No. F-6625. **PREPARE FRESH**. **PROTECT FROM LIGHT**.)

F. Orotate Reductase Enzyme Solution (Immediately before use, prepare a solution containing 1 - 2 units/ml of Orotate Reductase in cold Reagent A.)

PROCEDURE:

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

Deionized Water	6.40
Reagent A (Buffer)	10.00
Reagent B (Cys)	5.00
Reagent C (Orotic)	6.00
Reagent E (FAD)	0.10

Mix by stirring and adjust to pH 6.5 at 25°C with either 1 M HCl or 1 M NaOH.

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

	Test	Blank
Reaction Cocktail	2.75	2.75
Reagent F (Enzyme Solution)	0.05	------
Reagent A (Buffer)	------	0.05

Mix by inversion and incubate for 5 minutes at 25°C to activate the enzyme. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent D (ß-NADH)	0.20	0.20

Immediately mix by inversion and record the decrease in A_{340nm} for approximately 5 minutes. Obtain the ΔA_{340nm}/minute using the maximum linear rate for both the Test and Blank.

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CALCULATIONS:

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

3 = Total volume (in milliliters) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADH at 340 nm
0.05 = Volume (in milliliter) 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 convert 1.0 µmole of orotic acid to dihydroorotate per minute at pH 6.5 at 25°C.

FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 167 mM sodium phosphate, 67 mM cysteine hydrochloride, 2.0 mM orotic acid, 0.01 mM FAD, 0.11 mM ß-NADH and 0.05 - 0.1 unit orotate reductase.

REFERENCE:

Friedmann, H.C. and Vennesland, B. (1958) *Journal of Biological Chemistry* **233**, 1398-1406.

NOTES:

1. This assay is a modification of the enzyme assay described in the cited reference.

2. All product and stock numbers, unless otherwise indicated, are Sigma product and stock numbers.