

**Enzymatic Assay of SALICYLATE HYDROXYLASE
(EC 1.14.13.1)**

PRINCIPLE:

Salicylate + β -NADH + O₂ $\xrightarrow{\text{Salicylate Hydroxylase}}$ Catechol + β -NAD + H₂O + CO₂

Abbreviations used:

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

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

CONDITIONS: T = 30°C, pH = 7.6, A_{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

- A. 20 mM Potassium Phosphate Buffer with 1.0 mM Ethylenediaminetetraacetic Acid, pH 7.6 at 30°C (Prepare 100 ml in deionized water using Potassium Phosphate, Dibasic, Trihydrate, Sigma Prod. No. P-5504, and Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate, Sigma Stock No. ED2SS. Adjust to pH 7.6 at 30°C with 1 M HCl.)
- B. 3 mM Sodium Salicylate Solution (Salicylate) (Prepare 10 ml in Reagent A using Salicylic Acid, Sodium Salt, Sigma Prod. No. S-3007.)
- C. 5.4 mM β -Nicotinamide Adenine Dinucleotide, Reduced Form Solution (β -NADH) (Dissolve the contents of one 5 mg vial of β -Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt Stock No. 340-105, in the appropriate volume of cold Reagent A. **PREPARE FRESH.**)
- D. Salicylate Hydroxylase Enzyme Solution (Immediately before use, prepare a solution containing 0.25 - 0.5 unit/ml of Salicylate Hydroxylase in cold deionized water.)

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PROCEDURE:

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

	<u>Test</u>	<u>Blank</u>
Reagent A (Buffer)	2.70	2.70
Reagent B (Salicylate)	0.10	0.10
Reagent C (β-NADH)	0.10	0.10

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

Deionized Water	-----	0.10
Reagent D (Enzyme Solution)	0.10	-----

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

CALCULATIONS:

$$\text{Units/ml} = \frac{(r A_{340\text{nm}}/\text{min Test} - r A_{340\text{nm}}/\text{min Blank})(3.0)(df)}{(6.22)(0.10)}$$

3.0 = Total volume (in milliliters) of assay

df = Dilution factor

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

0.10 = Volume (in milliliters) of enzyme used

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

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

UNIT DEFINITION:

One unit will convert 1.0 μmole of salicylate and β-NADH to catechol and β-NAD per minute at pH 7.6 at 30°C.

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FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are
19 mM potassium phosphate, 0.97 mM
ethylenediaminetetraacetic acid, 0.1 mM sodium salicylate,
0.18 mM β -NADH, and
0.025 - 0.05 unit salicylate hydroxylase.

REFERENCE:

White-Stevens, R.H. and Kamin, H. (1972) *Journal of
Biological Chemistry* **247**, 2358-2370.

NOTES:

1. This assay is based on the cited reference.
2. Where Sigma Product or Stock numbers are specified,
equivalent reagents may be substituted.

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