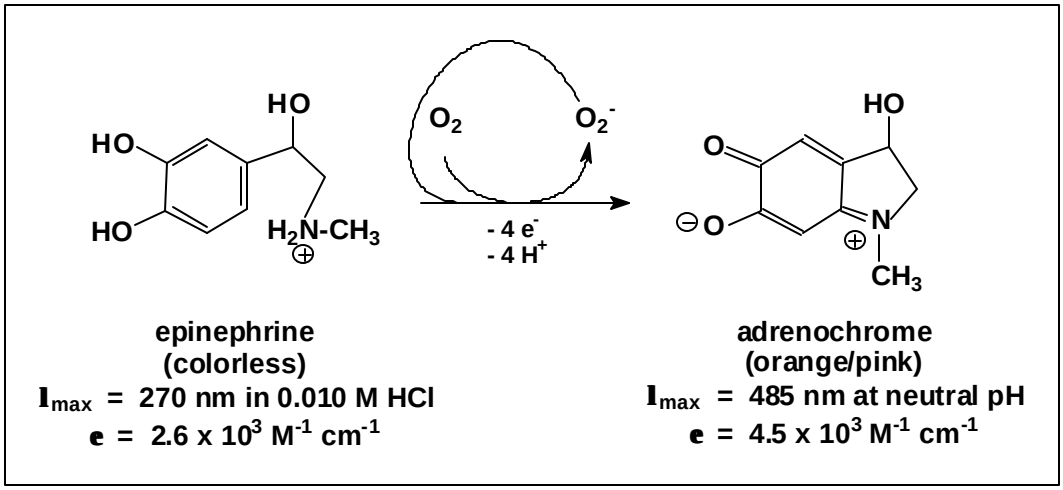
ASSAY FOR SUPEROXIDE DISMUTASE ACTIVITY USING THE ENZYME INHIBITION OF THE OXIDATION OF EPINEPHRINE©2000

GENERAL BACKGROUND:

Reaction catalyzed by SOD: $2\ O_2^- + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$ CuZnSOD = 3.15×10^4 g/mol

Substrate:	O_2^-	- superoxide anion (highly unstable in any form, for example: KO_2)
		- must be generated *in situ*
Products:	H_2O_2	- hydrogen peroxide
	O_2	- dioxygen

Indirect generation of O_2^-: Oxidation of epinephrine as shown below:



epinephrine (colorless)
λ_{max} = 270 nm in 0.010 M HCl
ε = 2.6 x 10^3 M^{-1} cm^{-1}

adrenochrome (orange/pink)
λ_{max} = 485 nm at neutral pH
ε = 4.5 x 10^3 M^{-1} cm^{-1}

Description of the assay:

The O_2^- substrate for SOD is generated indirectly in the oxidation of epinephrine at alkaline pH by the action of oxygen on epinephrine. As O_2^- builds in the solution, the formation of adrenochrome accelerates because O_2^- also reacts with epinephrine to form adrenochrome . Toward the end of the reaction, when the epinephrine is consumed, the adrenochrome formation slows down. If observed for long times, the adrenochrome disappears and brown, insoluble products form in the solution. (These brown products are closely related to the brown pigments in our skin and to the pigments that form when fruit is cut open and exposed to dioxygen.)

SOD reacts with the O_2^- formed during the epinephrine oxidation and therefore slows down the rate of formation of the adrenochrome as well as the amount that is formed. Because of this slowing process, SOD is said to inhibit the oxidation of epinephrine. The percent inhibition (%I) is hyperbolic with respect to the SOD concentration. This is contrary to the behavior of other enzymes, where a function related to their enzymatic activity is a linear function of the enzyme concentration.

Definition of assay terms:

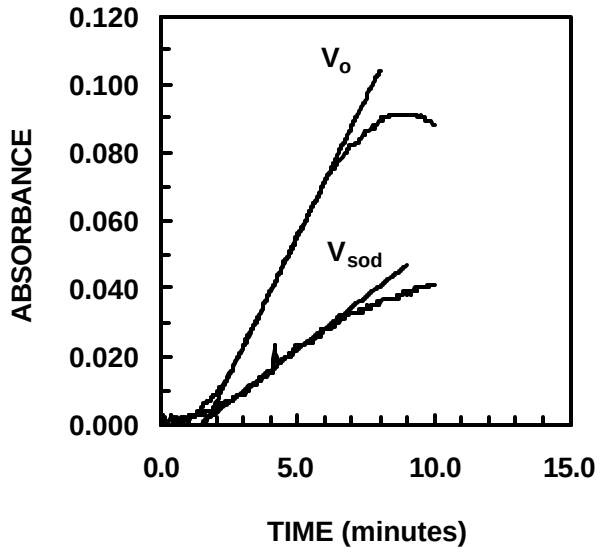
Kinetic run: Sigmoidal absorbance versus time curve

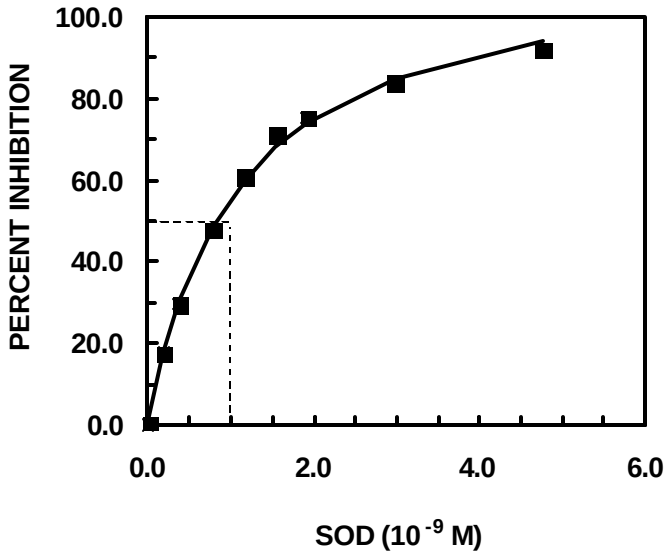
V_o = Slope of the linear portion of the absorbance versus time curve in the *absence* of SOD

$V_{s\ od}$ = Slope of the linear portion of the absorbance versus time curve in the *presence* of SOD

$$\%\ I = \frac{Vo - Vsod}{Vo} \times 100\% = \text{Percent Inhibition}$$

The percent inhibition is a hyperbolic function of the concentration of SOD.





SOD_{50} = Unit of SOD activity is defined as that amount of SOD required to cause 50% inhibition of the oxidation of the epinephrine (SOD_{50}). The SOD_{50} depends upon V_o (See Jewett SL, Rocklin, AM (1993). Variation of One Unit of Activity with Oxidation Rate of Organic Substrate in Indirect Superoxide Dismutase Assays. Anal. Biochem. 212: 555-559.)

STOCK SOLUTIONS:

A. 1.00 L of 0.020 M HCl

1.72 mL concd HCl (11.6 M) in 1.00 L
(for epi free base)

or 1.00 L of 0.010 M HCl
0.862 mL concd HCl (11.6 M) in 1.00 L
(for Epi•HCl)

B. 1.00 L of 0.050 M $NaHCO_3$-Na_2CO_3 buffer, pH 10.20 to 10.25 with 1.00 x 10^{-4} M EDTA

1. Clean a 1.00 L plastic storage bottle, cap, stir bar, and a 1500 or 2000 mL beaker carefully with cleaning solution. Rinse both thoroughly and carefully with double deionized water. Drain thoroughly.

2. Weigh out **0.03900 g** of $Na_2EDTA \cdot 2H_2O$ (FW = 372.2 g/mol) on the analytical balance. Quantitatively transfer the solid to the clean beaker.

   (1.00 L) (1.00 x 10^{-4} M) (372.2 g/mol) = 0.03722 g (or 37.22 mg)

3. Weigh **4.20 g** of $NaHCO_3$ (MW = 84.01 g/mol) on the top loading balance and quantitatively transfer the solid into the clean beaker.

   (1.00 L) (0.050 M) (84.01 g/mol) = 4.20g

4. Add about **800 mL of water** to the beaker along with a clean stir bar. Set beaker on stir motor at pH meter. Check the temperature of this solution. It should be 25° C.

5. **Standardize pH meter** at 25° C if it is not standardized.

6. Set **electrode** into buffer being sure not to batter the electrode with the stirring bar.

7. Add **concentrated NaOH** (**.** 3 M) until pH is 10.25.

8. Transfer to a **1.0 L volumetric flask** and make up to the mark.

9. **Remeasure pH** and adjust if necessary.

10. Transfer the buffer to the **clean plastic storage bottle with a clean cap**. Do not store this alkaline buffer in glass because silicates will be removed from the glass.

C. 25 mL of 0.0100 M epinephrine (MW = 183.2 g/mol) in 0.020 M HCl:
(epinephrine·HCl (MW = 219.7 g/mol)in 0.01 M HCl)**

1. The day before, clean a 25.00 mL volumetric flask with cleaning solution. Rinse thoroughly with double deionized water, drain and allow to dry completely. The epinephrine cannot be weighed into a wet container otherwise it will start oxidizing immediately.

2. Remove epinephrine from refrigerator or freezer. Allow it to come to room temperature without opening the vial (about 30 minutes). When finished, seal with parafilm and store with dessicant.

*******Wear gloves and a dust mask when weighing out the epinephrine*******

3. Weigh about **45.8 mg of epinephrine (free base)** (*or 0.054.92 g of epinephrine·HCl*) into the clean dry 25 mL volumetric flask. Record exactly the amount weighed out and proceed to step 3 immediately.

(0.025 L) (0.0100M) (183.2 g/mol) = 0.04580 g (or 45.80 mg)

*(0.025 L) (0.0100M) (219.7 g/mol) = 0.054920 g (or 54.92 mg)***

***Note: If you use the hydrochloride salt, weigh out 54.92 mg*

3. Immediately add **0.020 M HCl** to dissolve (*or 0.010 M HCl*). Do not wait any length of time to add the HCl because the epinephrine will oxidize. The epinephrine will dissolve quickly after the addition of the HCl.

4. Add the HCl to the mark. The final HCl will be 0.010 M (*or 0.010 M*) after the 0.010 M epinephrine free base reacts with the 0.020 M HCl (*or because the epinephrine is already protonated*).

5. Calculate the molarity from the molecular weight and the 25.00 mL volume. Example:

$$0.04462\ \text{g} \times \frac{1\ \text{mol}}{183.2\ \text{g}} \times \frac{1}{0.02500\ \text{L}} = 0.00974\ \text{M}$$

6. Measure the spectrum of a sample by diluting 20 ：L into a final volume of 1000 uL with 0.10 M HCl. Take the spectrum from 240 nm to 340 nm (or 250 nm to 350 nm) to verify that the concentration is correct.

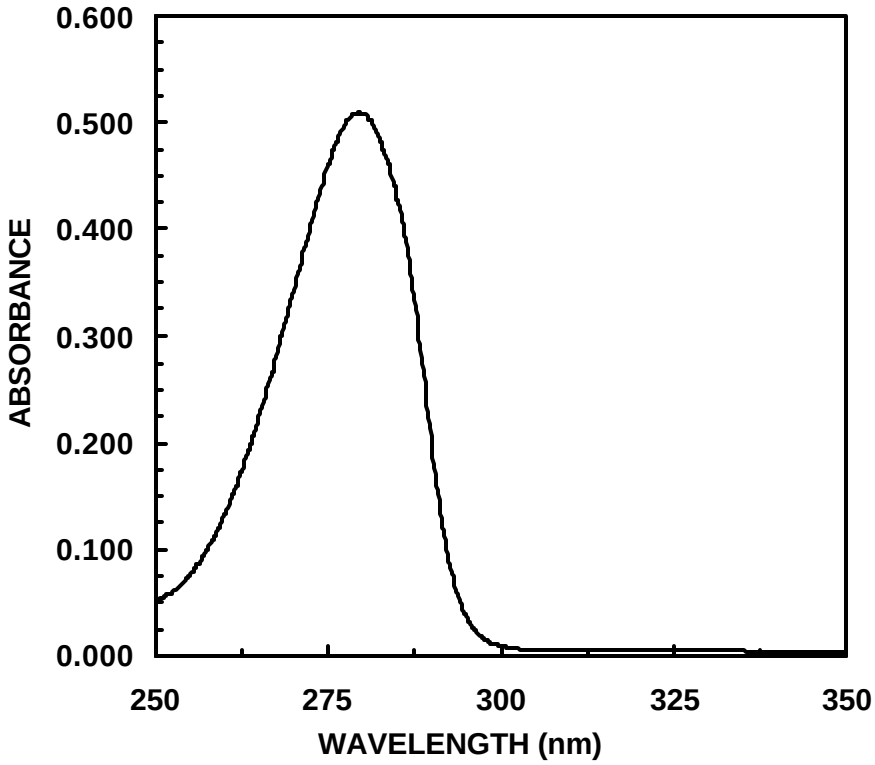
Example: the expected absorbance for a ~ 2 x 10^{-4} M solution would be expected to be ~ 0.50

$$\frac{(20\ \text{：L})(0.00974\ \text{M})}{1000\ \text{：L}} = 1.95 \times 10^{-4}\ \text{M}$$

Expected absorbance at 258 nm from Beer's law:

$A = \varepsilon c l$ where $\varepsilon = 2.6 \times 10^{3}\ M^{-1} cm^{-1}$ and $l = 1.0$ cm

$= (2.6 \times 10^{3}\ M^{-1} cm^{-1}) \times (1.95 \times 10^{-4}\ M) \times (1.0\ cm) = 0.51$



PREPARATION OF SOD STANDARD:

PREPARATION OF STOCK SOLUTION: The stock solution of standard SOD is usually the remaining stock solution or a control solution used for an experiment in progress. If such a solution is unavailable, then a sample of SOD must be weighed out and taken up in millipore-filtered buffer (mpf buffer).

Typical buffers (with ≈ about 0.10 M):

0.050 M TRIS-HCl, 1.0 x 10^{-4} M EDTA, at pH 7.4 with NaCl for ≈ = 0.10 M
0.050 M MOPS, 1.0 x 10^{-4} M EDTA, at pH 7.4 with NaCl for ≈ = 0.10 M
0.050 M phosphate, 1.0 x 10^{-4} M EDTA, at pH 7.4, ≈ ~ 0.10 M

Example #1: 16 mg sample of SOD with sucrose from a sealed vial
(Oxis International www.oxis.com)

1. Take the sealed vial out of the freezer and set on ice.
2. Millipore filter about 10 - 20 mL of buffer ("mpf buffer"). You will need 4.5 mL.
3. Carefully open the seal and remove the plastic stopper.
4. Pipet 4.5 mL of mpf buffer into the vial and replace plastic stopper immediately.
5. Mix gently to dissolve the SOD. Remove plastic stopper, replace it, and mix again. Any solid that adheres to the stopper must be dissolved before proceeding.

$$\frac{16\ \text{mg}}{4.5\ \text{mL}} = \frac{3.55\ \text{mg}}{\text{mL}} \times \frac{1\ \text{mol}}{3.15 \times 10^{4}\ \text{g}} = 1.13 \times 10^{-4}\ \text{M (by weight)}$$

6. Store this solution refrigerated. When in use, always store on ice.
7. The solution stability is unknown. Because of the sucrose, bacteria will grow in it. It can be stabilized by dialysis into buffer to remove the sucrose.

Example #2: Weigh out lyophilized SOD from Oxis International. This is about $4 a mg (2/2000) so the quantitiy above is expensive, but convenient. Cut down the weight and add buffer to give ~ 1.0 mg/mL solution (~ 30 ≈M) to avoid the second dilution indicated below.

DETERMINATION OF CONCENTRATION OF STOCK SOLUTION OF SOD

The CuZnSOD has an extinction coefficient at 258 nm of 1.03 x M^{-1} cm^{-1}. If the absorbance of the stock solution is measured directly, it will be above 1.0 absorbance unit where most instruments are less accurate. It is recommended that a dilution is made in order to have a more accurate measure of the stock concentration

1. Make a dilution of the SOD as follows for a 1.13 x 10^{-4} M solution (modify recipe according to the stock concentration):

   300 ∶L SOD stock solution
   600 ∶L of buffer
   ---
   900 ∶L total

   $$\text{SOD in dilution} = \frac{300\ \text{∶L}}{900\ \text{∶L}} \times 1.13 \times 10^{-4}\ \text{M} = 0.336 \times 10^{-4}\ \text{M}$$

2. Measure spectrum from 240 nm to 340 nm, being sure to do baseline properly with buffer in cuvet(s). Determine the absorbance at 258 nm. If the absorbance at 340 in the spectrum is not zero, subtract the A_{340} from the A_{258} before determining the cuvet SOD concentration:

   A_{258} = 0.357
   A_{340} = - 0.010

   A_{258} corr = 0.347

   $$\text{SOD in cuvet} = \frac{0.347}{1.03 \times M^{-1}\ cm^{-}} = 3.47 \times 10^{-5}\ \text{M}$$

   $$\text{SOD in stock} = \frac{900\ \text{∶L}}{300\ \text{∶L}} \times 3.47 \times 10^{-5}\ \text{M} = 1.04 \times 10^{-4}\ \text{M}\ \text{(By uv)}$$

   Note: The uv concentration is usually about 80% of the concentration by weight in lyophilized samples.

3. Diagnostic for the purity of the superoxide dismutase:

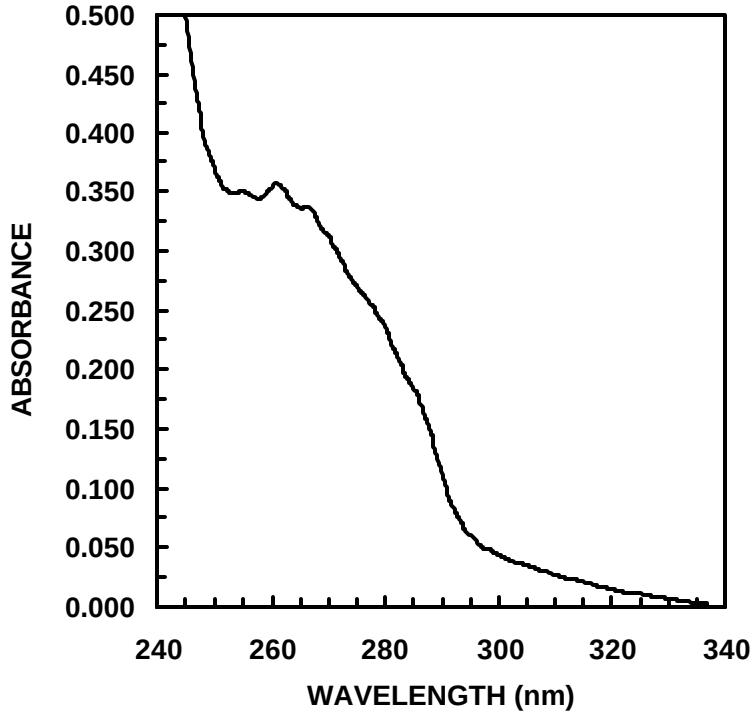
   Obtain the absorbance at 280 nm (subtrace the absorbance at 340 if necessary as above) and find the following ratio A_{258}/A_{280}. This ratio should be above 1.5.

   Sample calculation:

   A_{258} = 0.357
   A_{280} = 0.213
   A_{340} = 0.010

   A_{258}corr = 0.357 - 0.010 = 0.347
   A_{280}corr = 0.213 - 0.010 = 0.203

   $$\frac{A_{258}\text{corr}}{A_{280}\text{corr}} = \frac{0.347}{0.203} = 1.71$$



PREDILUTION OF SOD FOR KINETIC RUNS (immediately before use):

These dilutions are made immediately before use in the reaction mixtures. They are used for several hours and discarded when all assays have been performed

This assay is extremely sensitive to SOD. It requires prediluting the SOD to ~ 10^{-8} M range, then using portions of this solution for the kinetic runs. Trial and error may have to be used for getting the optimal data. The predilutions below provide a sample based on the #1 stock solution shown above:

1st dilution

$$\frac{45\ \mu L}{4000\ \mu L} \quad \frac{3955\ \mu L}{4000\ \mu L} \quad \frac{(45\ \mu L)(1.13 \times 10^{-4}\ M)}{4000\ \mu L} = 1.20 \times 10^{-6}\ M$$

2nd dilution

$$\frac{25\ \mu L}{1000\ \mu L} \quad \frac{975\ \mu L}{1000\ \mu L} \quad \frac{(25\ \mu L)(1.20 \times 10^{-6}\ M)}{1000\ \mu L} = 3.00 \times 10^{-8}\ M \ (30.0\ nM)$$

A 2nd dilution predilution of the SOD to 3.00×10^{-8} M would dictate a typical minimum set volumes (see below) for the determination of the unit of SOD activity, or SOD_{50}, in a 1.000 mL final volume for the kinetic

Volumes of 30 nM SOD	**SOD nM in cuvet**	**Comments**
0 µL	0.00	For V_o determination
7 µL	0.21	Less than 50% inhibition
14 µL	0.42	"
21 µL	0.63	Greater than 50% inhibition
28 µL	0.84	"

A minimum set of 4 SOD concentration,
two giving < 50% inhibition and two giving > 50% inhibition,
are required to accurately determine SOD_{50}.

The SOD_{50} is dependent upon V_o such that the amount of SOD increases (sensitivity goes down) as V_o increases. V_o values of 0.010 min^{-1} provide a compromise between sensitivity and faster rate. The protocol at the right is recommended for $V_o = 0.010 \pm 0.001$ min^{-1} for a SOD_{50} of ~ 0.85 nM

If $V_o = 0.006 \pm 0.001$ min^{-1}, the SOD_{50} is ~ 0.50 nM

PROCEDURE FOR SETTING UP KINETIC RUNS:

1. Consult the instrument manual and set kinetics mode at 485 nm, 0.0 - 0.10 absorbance scale. Set T = 25 °C. The total time for a kinetic run will be 10-12 min for V_o determinations and up to 20 min for the inhibited runs with SOD. The time can be adjusted as the SOD is increased.

2. Equilibrate a portion of the pH 10.2 buffer to 25° C (~1 mL per kinetic run x # runs anticipated + small excess). Check the temperature of this buffer solution with a clean thermometer. Shake vigorously several times to equilbrate with O_2 after it has reached 25 °C.

3. **Preparation of Standard Curve** (Percent Inhibition versus SOD):

   A. Determination of V_o (uninhibited kinetic runs - do as many times as necessary to obtain reproducible results):

   The order of addition and mixing are critical to reproducibility

   (a) Pipet the following directly into cuvet:

   925 : L 0.050 M HCO_3^-/CO_3^{2-} buffer pH 10.25 (order):
   50 : L buffer (same as buffer in which SOD standard is made)

   (b) Mix and zero the spectrophotometer.

   (c) Add 25 : L 0.0100 M epinephrine, mix immediately, and start recording absorbance at 485 nm versus time (0.0 to 0.10 scale).

   (d) Determine the slope of the linear portion of this sigmoidal plot. It should be 0.010 ± 0.001 min^{-1}. If not, adjust the epinephrine volume to achieve a V_o in this range.

   (e) Repeat the determination of V_o until proficiency and a high degree of reproducibility is achieved. The % deviation of the runs should not exceed 5%. A variation of 5 - 10% is acceptable, however, the reliability is compromised.

B. Determination of V_{sod} (inhibited kinetic runs - do as many times as necessary to obtain reproducible results):

(a) Pipet the following directly into cuvet:

925 ΅L 0.050 M HCO_3^-/CO_3^{2-} buffer pH 10.25 (order)
50 ΅L sum of SOD +enzyme buffer (same as buffer in which SOD standard is made see table below)

Example:

Volume of 30 nM SOD	Volume of enzyme buffer	SOD nM in cuvet	
0 ΅L	50 ΅L	0.00	For determination of V_o; see Part A above
7 ΅L	43 ΅L	0.21	
14 ΅L	36 ΅L	0.42	
21 ΅L	29 ΅L	0.63	
28 ΅L	22 ΅L	0.84	

NOTE: A minimum set of 4 SOD concentration, two giving < 50% inhibition and two giving > 50% inhibition, are required to accurately determine SOD_{50}.

(b) Mix and zero the spectrophotometer.

(c) Add 25 ΅L 0.0100 M epinephrine (or adjusted volume determined in Part A above), mix immediately, and start recording absorbance at 485 nm versus time (0.0 to 0.10 scale).

(d) Determine the slope of the linear portion of this sigmoidal plot. (*See NOTE below)

(f) Determine each V_{sod} in duplicate or more, however many times it takes to achieve a deviation of # 5%. A deviation of 5 - 10% is acceptable, however, the reliability is compromised.

*NOTE: Set up the next kinetic run before analyzing the kinetic run just obtained. It is always a good practice to determine the V values during the assay. If adjustments to the volumes need to be made, they can be made on the spot. Also, reproducibility is monitored continuously.

C. MULTICUVETS: Use of 2 or more cuvets in same kinetic run.

DO NOT CROSS CONTAMINATE SOLUTIONS DURING THE PIPETTING

Sample procedure for 6 cuvets: "simultaneous" duplicate runs for V_o, $V_{SOD\ 7\ \mu L}$, $V_{SOD\ 14\ \mu L}$

1. Place a reference cuvet in the spectrophotometer with pH 10.25 carbonate buffer.
2. Pipet 925 ΅L of the pH 10.25 carbonate buffer into 6 cuvets in the multisample compartment.
3. Pipet 50 ΅L of buffer for the enzyme into cuvets #1 and #2 (V_o). Mix. (Vol = 975 ΅L)
4. Pipet 43 ΅L of 30 nM SOD into cuvets #3 and #4. Mix. (Vol = 975 ΅L)
5. Pipet 36 ΅L of 30 nM SOD into cuvets #5 and #6. Mix. (Vol = 975 ΅L)
6. With the spectrophotometer reading cuvet #1, press autozero (and/or cell blank).
7. Take up 25 ΅L of the 0.010 M epinephrine into a pipet tip. Pick up the stirrer with your left hand; pipet the 25 ΅L into cuvet #1, mix immediately.
8. Repeat step 7 for all cuvets as quickly as possible, using a clean pipet tip each time. The addition of the epinephrine to all 6 cuvets, should not take longer than 1 minute.
9. Start recording absorbance at 485 nm versus time.

DATA ANALYSIS:

Consult sample spreadsheet and sample hyperbolic and inverse plots before proceeding with these instructions.

1. Using a linear regression program, calculate the slopes of the linear portion of absorbance versus time plots (see page 2 for sample plot). Enter these values and the corresponding SOD concentrations into EXCEL spreadsheet. Do *not* calculate V_{SOD}/V_o as shown on the sample spreadsheet. This ratio decreases with increasing SOD and is used to *illustrate* the inhibition of epinephrine oxidation by SOD.

2. Calculate percent inhibition and plot this versus SOD concentration. These data fit a hyperbolic curve (see page 2 as well as PDF file) indicating that the assay becomes less sensitive to SOD concentrations at higher SOD. Use the slope and y-intercept parameters from the double reciprocal plot to obtain a theoretical line for data.

3. Calculate inverse percent inhibition and inverse SOD concentration and plot these to obtain the slope and the y-intercept (see sample in PDF file). Use these parameters to calculate a theoretical line for the inverse plot and the hyperbolic plot.

4. Summary of equations needed to analyze Standard SOD data:

$$\%\ I = \frac{V_o - V_{sod}}{V_o} \times 100\% = [\text{SOD nM}] / \{ m + b^* [\text{SOD nM}]) = \text{hyperbolic dependence}$$

$$\text{Inverse \% I} = m\,(1/[\text{SOD nM}]) + b = \text{linear dependence}$$

$SOD_{50} = 50m / (1 - 50b)$ = SOD (nM) required to cause 50% inhibition.
$SOD_{50}' = m/b$ = SOD (nM) required to cause half-maximal inhibition.

5. For cell extracts, the equations are of similar form but with ∶L as the x variable rather than SOD (nM):

$$\%\ I = \frac{V_o - V_{ext}}{V_o} \times 100\% = [\text{∶L ext}] / \{ m + b^* [\text{∶L ext}]) = \text{hyperbolic dependence}$$

$$\text{Inverse \% I} = m'\,(1/[\text{∶L ext}]) + b' = \text{linear dependence}$$

$EXT_{50} = 50m' / (1 - 50b')$ = Extract volume (∶L) required to cause 50% inhibition
$EXT_{50}' = m'/b'$ = Extract volume (∶L) required to cause half-maximal inhibition. (Used if maximum % I < 90%)

6. Assuming the EXT_{50} (∶L) has an amount of SOD that results in the SOD_{50} (nM) concentration in the 1.00 mL final volume in the cuvet, then the SOD concentration (nM) in the extract can be determined as follows:

Equality: EXT_{50} (∶L) = SOD_{50} (nM)

$$\text{Conversion factor: } \frac{SOD_{50}\ (nM)}{EXT_{50}\ (∶L)}$$

$$1000\ ∶L \times \frac{SOD_{50}\ (nm)}{EXT_{50}\ (∶L)} = \text{nM of SOD in extract}$$

7. The SOD in a cell extract is the total SOD determined by the activity of both the CuZnSOD (cytosolic) and the MnSOD (mitochondrial) enzymes. To obtain the contribution from the MnSOD, the CuZnSOD activity is suppressed with 1.00 mM NaCN in the assay (see separate sheet on its preparation). The V_{sod} values obtained obtained in the presence of cyanide are then used to find a new EXT_{50} in the presence of cyanide and attributable to the MnSOD. Both enzymes react with the same rate constant with O_2^- so they are identical in their ability to inhibit oxidation of epinephrine.

   Sample data analysis to obtain % CuZnSOD and % MnSOD: In extract:

   SOD_{50} = 0.50 nM Total SOD = 50 nM = (1000 ∶L x 0.50 nM)/10 ∶L

   $EXT_{50}(-CN^-)$ = 10 ∶L = 0.50 nM
   $EXT_{50}(+CN^-)$ = 50 ∶L = 0.50 nM MnSOD = 10 nM = (1000 ∶L x 0.50 nM)/50 ∶L

   By difference, CuZnSOD = 50 nM - 10 nM = 40 nM Percents:
   CuZnSOD = 80% = (40 nm / 50 nm) x 100%
   MnSOD = 20% = (10 nm / 50 nm) x 100%

8. For dismutase samples that have undergone inactivation, the SOD_{50} shifts to larger values (see sample PDF file). This indicates that it takes more of the sample because some of the SOD has been inactivated.