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Oxygen Uptake and Spectrophotometric Measurement of Superoxide, Hydrogen Peroxide and Hydroxyl Radicals

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Introduction

The uptake or evolution of oxygen during a reaction can be monitored by use of a Clark-type electrode. The method is particularly useful when turbidity or interfering chromogens preclude the use of spectrophotometry [1].

Protocol

Electrode calibration

1. Add deionized water (2 mL) to the temperature-controller reaction chamber with magnetic stirring, and apply 0.7 V across the electrodes.
2. An output of 0.1 to 10 mV is used with a chart recorder set to a value of 100%.
3. Leave electrode to settle for 5 min.
4. Add a few crystals of sodium dithionate to remove O_2.
5. This value represents 0% O_2. The electrode is now calibrated from 0 to 100% oxygen.
6. Apply test samples to chamber after carefully washing each time.

Calculation

The concentration of oxygen can be calculated by use of the information that air-saturated water at 30°C contains 0 .4 60 µml O_2.

Spectrophotornetric Measurement of Superoxide Anion Radicals

First applied to neutrophil studies in 1968, the nitroblue tetrazolium (NBT) test provides a simple assay for $\cdot O_2^-$ production. NBT is reduced by O_2^- to a blue formazan product which is measured at 620 nm. Spectrophotometric assay techniques are covered in full [2,3]. Pulse radiolysis techniques are also described [4].

Spectrophotornetric Measurement of Hydrogen Peroxide

Any system producing $\cdot O_2^-$ will also produce H_2O_2 by the dismutation reaction. H_2O_2 is an uncharged molecule that can readily enter cells as a water look-alike. Most assays utilize a peroxidase with coupled oxidation of a detector molecule.

H_2O_2 can also be determined by measuring the loss of scopoletin fluorescence (460 nm), an assay covered in detail [5] or on the basis of its reaction with cytochrome-c peroxidase to form a stable complex with absorbance at 419 nm (A_{419}) [1]

Herein we describe a method using phenol red to measure hydrogen peroxide in breath condensates [6].

Protocol

1. Dilute stock hydrogen peroxide (30% v/v) 1:1000 with Chelex resin treated distilled water.
2. Calculate the concentration of H_2O_2 by measuring absorbance at 240 nm using a molar absorption coefficient of 43.6 M/cm.
3. Prepare buffered phenol red-peroxidase reagent containing NaCl (40 mM), potassium phosphate (pH 7.0, 10 mM), phenol red (0.1 g/L), and horseradish peroxifase (HRPO; 8.5 unit/mL), adding phenol red and HRPO to buffer just before use.
4. To 1.0 mL phenol red reagent add 0.5 mL breath condensate. Hydrogen peroxide standards are similarly treated.
5. Mix and stand for 5 min at 25°C.
6. Add NaOH (1 M, 10 µL) to give a pH of 12.5 and a stable purple color.
7. Measure absorbance at 610 nm.

Calculation

Include a blank containing H_2O_2-free water and calculate values from an H_2O_2 standard curve.

Spectrophotometric Measurement of Hydroxy Radical Damage

The hydroxyl radical is much too reactive to be measured directly by simple spectrophotometric techniques. Evidence characteristic of its damage is, therefore, usually sought by use of a detection molecular for such damage. Here we describe the use of the sugar 2-deoxy-D-ribose [7-9].

Protocol

1. Into clean glass tubes place 5-deoxy-D-robose (5 mM, 0.2 mL) and sodium phosphate buffer (0.024 M containing 0.15 M NaCl, pH 7.4, 0.2 mL.
2. Add $OH^\cdot$ generating system, i.e.;

[A] 0.1 mL of ferrous salt (freshly prepared at pH <6.0) or

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[B] ferric-EDTA + ascorbate or

[C] hypoxanthine + xanthine oxidase + ferric salt.

3. Incubate reactants for 30 min at 37°C.
4. Add thiobarbituric acid (1% w/v, 0.5 mL) and trichloroacetic acid (2.8% W/V, 0.5 mL).
5. Heat tube contents at 100°C for 10 min.
6. Cool and read absorbance at 532 nm or fluorescence at 553 nm after excitation at 532 nm.

Calculation

The assessment of $OH^{\cdot}$ damage can be used to test the effectiveness of scavengers and inhibitors, and to determine second-order rate constants for reaction of $OH^{\cdot}$ with various scavenger.

Comments

$OH^{\cdot}$ generating systems [A] and [B] are not inhibited by SCD, whereas [C] is. All three $^{\cdot}OH$-generating ststems should be inhibited by catalase or other H_2O_2 renoving enzymes. If silutions are turbid extract chromogen into butan-1-ol.

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