NSMRL TECHNICAL REPORT M87-3

SUPEROXIDE DISMUTASE ASSAYS

by

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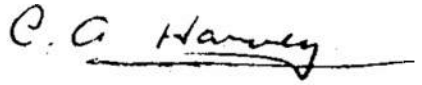

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ABSTRACT

A number of studies support the idea that superoxide dismutase is one of the enzymes that modulates the threat of oxidative stress. This technical report presents detailed instructions for two methods presently being used at the Naval Submarine Medical Research Laboratory (NSMRL) to assay for superoxide dismutase. Both assays are of the indirect type consisting of two components: a superoxide generator and a superoxide detector. In the first method the generator produces the radical at a controlled rate, and in the absence of superoxide dismutase (SOD), the radical produced reacts with the detector. A unit of SOD then has been defined as that amount which will reduce the rate produced by the generator to 50% of its control value. The second method, which is about 100-fold more sensitive, takes advantage of the biphasic nature of the production of the radical and its dismutation both spontaneously and by SOD. By allowing the generator to react for a specific time period before addition of the detector, a burst of reduction is obtained followed by a linear rate after the detector reaches a steady state. The height of this burst in the presence and absence of SOD thereby gives a sensitive assay for which the unit (50% of control burst) is in the picomolar range. These assays are useful in determining SOD in a variety of tissue types such as erythrocytes obtained from Navy divers or cell culture samples that have been exposed to oxidative stress.

TABLE OF CONTENTS

I. INTRODUCTION

A blue copper-containing protein called hemocuprein was first isolated in 1939 from bovine erythrocytes. Subsequently, other copper proteins were isolated from human blood and other tissues. Eventually these purified proteins were called "erythrocuprein" although no activity was ascribed to them until 1969 when McCord and Fridovich (1) showed an identity of erythrocuprein with an enzymic activity which rapidly catalyzed the dismutation of superoxide free radical anions in the reaction:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow[SOD]{\text{enzyme}} O_2 + H_2O_2$$

Hence these proteins were renamed superoxide dismutases (SOD) and shown to be very important in many aspects of the regulation of oxygen toxicity.

Quantitative assays of superoxide dismutase activity have had a number of difficulties. For one thing, neither the product nor the substrate are simple to detect. For another, the substrate is short lived and readily reacts with many factors in solution as well as, frequently, the vessel itself. A standard assay was described by McCord and Fridovich in their original paper (1), and this assay has continued to be the one of choice for many investigators. These assays have been of the indirect type consisting of two components: a superoxide generator and a superoxide detector. The generator produces the free-radical at a controlled rate and in the absence of superoxide dismutase the radical produced reacts with the detector. A unit of SOD then has been defined as the amount which will reduce the rate of radical produced by the generator to 50% of its control value.

A more sensitive method described by Kirby and Fridovich (2), takes advantage of the biphasic nature of the production of the radical and its rate of dismutation both enzymically and spontaneously. By allowing the generator reaction to proceed for a specific time period before the addition of the detector, a burst of reduction is obtained followed by a linear rate after the detector reaches a steady state. The height of this burst in the presence and absence of SOD thereby gives a sensitive assay for which the unit of activity is in the picomolar range. Both of these assay methods are outlined in this report and modifications introduced.

II. REAGENTS FOR THE SOD ASSAYS

See the Appendix for sources of reagents. Our assays are based on a 3 ml final volume for spectrophotometry. We use 18 megohm water for all stock solutions and buffers.

1. Buffer for the standard assay (Phosphate according to reference #3)

500 ml of 50 mM potassium phosphate at pH 7.8 and 0.1 mM EDTA is prepared as follows and store at 25° C

STOCK A: Monobasic KH_2PO_4 100 mM stock solution
M. W. 136.09 dissolve 6.805 gm/500 ml H_2O

STOCK B: Dibasic K_2HPO_4 100 mM stock solution
M. W. 174.18 dissolve 8.709 gm/500 ml H_2O

STOCK C: EDTA 100 mM stock solution
dissolve 1.86 gm/500 ml H_2O

For working buffer mix 210 ml Stock A
229 ml Stock B
5 ml Stock C
qs to 500 ml H_2O

2. Buffer for the sensitive assay (Carbonate according to reference #2)

500 ml of 15 mM sodium carbonate at pH 10.2 and 0.1 mM EDTA is prepared as follows and store at 25° C

STOCK A: Sodium Carbonate 30 mM stock solution
M. W. 106 dissolve 1.59 gm/500 ml H_2O

STOCK B: Sodium Bicarbonate 30 mM stock solution
M. W. 84 dissolve 1.26 gm/500 ml H_2O

STOCK C: EDTA same as above

For working buffer mix 165 ml Stock A
85 ml Stock B
5 ml Stock C
q.s. to 500 ml H_2O

3. Cytochrome C stock for both standard and sensitive assays (2.0 mM in water) M.W. 12,384 (conc. can be checked with E 550).

Dissolve 50 mg in 2 ml of water and divide into 4 tubes of 0.5 ml each, store frozen

a) For the standard assay thaw, add 9.5 ml PO_4 buffer and this gives 0.1 mM to use 0.3 ml per assay, ie., = 30 assays.
b) For the sensitive assay thaw and use without dilution 30 ul directly, i.e., = 16 assays.

4. Xanthine stock for both assays (0.5 mM in Buffer)
M.W. 152 (not very soluble, dissolve and dilute carefully)

Dissolve 7.6 mg in 10 ml of PO_4 Buffer[1] and divide into 20 tubes of 0.5 ml (a 5 mM stock) store at 25°C

a) For the standard assay add 4.5 ml PO_4 Buffer. This gives 0.5 mM to use 0.3 ml per assay = 15 assays
b) For the sensitive assay use 30 ul per tube directly

5. Xanthine Oxidase stock for both assays

For both assays the concentration is adjusted to give a reaction rate so that the change at 550 nm is 0.02 to 0.04 OD/min.

Stock of Xanthine Oxidase EC 1.1.3.22 is at 25 U in 0.5 ml and is stored at 4°C

Dilute 50 ul + 4.95 ml PO_4 Buffer[1] and divide into 10 tubes of 0.5 ml and store frozen.

a) For standard assay dilute 0.5 ml + 4.5 po_4 buffer use 10 ul per tube.
b) For sensitive assay use 30 ul per tube.

6. Superoxide dismutase standard stock

Stock of superoxide dismutase EC 1.15.1.1 is at 30,000 units in 1.5 ml and is stored at 4°C.

Dilute 30 ul +2.97 ml PO_4 Buffer[1] and divide into 6 tubes of 0.5 ml and store frozen.

a) For use in standard assay dilute (i) 30 ul + 2970 ul Buffer[1] and serially dilute (ii) 30 ul + 270 ul Buffer[1].
b) For use in sensitive assay dilute (i) 30 ul + 2970 ul Buffer and serially (ii) 30 ul + 2970 ul Buffer

7. Nitroblue Tetrazolium stock

To 10 mg of anhydrous material add 2.5 ml Buffer.

III. METHODS FOR THE SOD ASSAYS

Both of these methods are based on (i) a superoxide generator (xanthine plus xanthine oxidase), (ii) a spectrophotometric detector (reduction of cytochrome C) and (iii) for the standard assay, the inhibition of the rate of scavenging of the radical by the superoxide dismutase, or (iv) in the sensitive assay the dimunution of the burst of superoxide generated after a time interval (10 minutes) by the presence of the superoxide dismutase.

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[1] In our modification from this study we use the carbonate buffer at pH 10.2 to avoid the solubility problem.

For the standard assay (according to reference 3)

To 2.4 ml of PO_4 Buffer at 25°C (as a control or containing a standard SOD or unknown)
add 0.3 ml cytochrome C (at 0.1 mM)
and 0.3 ml xanthine (at 0.5 mM)
at T=0 add 10 ul Xanthine Oxidase (at 7.5 mU)
Read absorbance at 550 nm
for 3 minutes. Control rate (no SOD) should give 0.02 to 0.04 O.D./min.
"one unit of SOD = amount to reduce control rate by 50%"

For our modification of the standard assay in this study

To 2.9 ml of Carbonate Buffer at 25°C
add 30 ul cytochrome C (at 1.0 mM as in sensitive assay) and 30 ul Xanthine (at 5 mM in carbonate Buffer)
at T = 0 add 30 ul Xanthine Oxidase
Read absorbance at 550 nm for 3 minutes

For the sensitive assay (according to reference 2)

As a control, to 2.9 ml of Carbonate Buffer at 25°C, add 30 ul of Xanthine stock and add 30 ul of Xanthine Oxidase. Cover with parafilm and invert twice.

Incubate 10 minutes at 25°C.

At the end of 10 minutes add 30 ul Cytochrome C invert twice and read at 550 nm.

Compare reading at T=0 to T=10 mins (no SOD)

This is the burst size e.g. .300 O.D. units (10 mins)
minus .225 " " (0 mins)
B_o burst size equals .075 O.D. units

$$\text{units} = \frac{B_o}{B_i} - 1$$

where B_o and B_i are respectively the burst size without and with SOD.

"one unit of SOD = amount to reduce the burst size by 50%"

For Standard SOD graph

Use 10, 20, 30, 40 and 50 ul volumes of diluted SOD stock.

IV. OPERATION OF THE PERKIN-ELMER LAMBDA 7 SPECTROPHOTOMETER AND SUPER SIPPER

A. Start up

1. Uncover instrument and turn on main power of spectrophotometer and leave on 5 minutes for warm up. Reads READY on cathode ray tube, CRT.

2. Record in log book and date and time on page 1 line 1 of CRT.
3. Tighten tubing on super sipper, put clean water under sampling tube and turn on power (on back right)
4. Press sample activator button and run water through cell, 3 rinses at sample volume 10 plus 3 rinses in Buffer at setting 3.
5. Check the correct page and set method of spectrophotometer for TIME DRIVE line 2 page 2, for SCAN line 3 page 1 of CRT.
   NOTE: During operation do not change from TIME DRIVE to SCAN without obtaining hard copy of data page
6. Set up method on CRT (page 2 for TIME or SCAN).
7. Set "next sample" at 1 on page 2 (DATA), page 3 is clear.
8. Wait 30 minutes and run background check (activate twice for Bkg/Corr or Run.
9. Run samples (use only visible lamp if UV is not needed)

B. Shut down
1. Turn off main power of spectrophotometer
2. Run clean water through super sipper 3 times at volume 10
3. Loosen tubing on super sipper and turn off. Leave full beaker of water to siphon through sampling tube overnight.
4. Cover instrument

V. RESULTS

A. Setting the spectrophotometer to examine the superoxide generator

Xanthine and Xanthine Oxidase as used in the assay for the superoxide generator were examined in the ultraviolet region of the absorption spectrum to observe the reaction. The spectrophotometer was used by setting the method mode of the instrument to SCAN mode from 200 to 320 nm. The sample was scanned 5 times at 3 minute intervals. Settings on the Perkin-Elmer Lambda 7 are shown in Figure 1.

B. Results of the scan of the superoxide generator

The scan 5 times at the spectrophotometer settings of Figure 1 giving the absorbance as a function of wavelength for the xanthine-xanthine oxidase generator are shown in Figure 2. Scan number 1 illustrates the spectral pattern of xanthine with an absorbance peak at 272 nm. The reaction continued with another scan every 3 minutes until the spectral pattern of uric acid predominated with absorbance peaks at 291 and 233 nm. By the fifth scan the conversion of xanthine to uric acid by xanthine oxidase was beginning to slow down. The data for these SCANS given on the Results page of CRT are in Figure 3.

C. Setting the spectrophotometer to examine the superoxide detector

Cytochrome C as used in the assay as the superoxide detector was examined in the visible region of the absorption spectrum to observe the reaction. The spectrophotometer was used by setting the method mode of the instuemnt to scan mode from 500 to 600 nm. The sample was scanned 5 times at 3 minute intervals. Settings on the METHOD page on the Perkin-Elmer Lambda 7 are shown in Figure 4 for these scans.

D. Results of the scan of the superoxide detector

The scan 5 times of the spectrophotometer settings of Figure 4 gave the absorbance as a function of wavelength for the cytochrome C detector shown in Figure 5. Scan number 1 illustrates the spectral pattern of cytochrome C before reduction by the xanthine-xanthine oxidase superoxide generator with an absorbance peak at 528 nm. The reaction continued with another scan every 3 minutes until the spectral pattern of the oxidized Cytochrome C predominated with absorbance peaks at 550 and 520 nm. The rate of the reaction was measured by selecting the change at 550 nm (literature reference 1). The data for these scans given on the RESULT page of the CRT are in Figure 6. The peak rising at 550 nm is used for the rate determinations of the next sections (E, F and G).

E. Setting the spectrophotometer for the standard superoxide dismutase assay.

For the standard assay described in III (Methods) the instrument was set on TIME DRIVE as shown in Figure 7, except only the data for the first cycle and only the Results page were needed for calculations.

F. Results of the control run for the standard superoxide dismutase assay.

The graphic reaction rate and the Results page at the settings cited in Figure 7 are presented in Figure 8 for the control run (no SOD standard or unknown added). For the standard assay the slope of the reaction curve (rate) is used to determine the percent reduction of the slope produced by added SOD. In the control run shown in Figure 8 the slope does not decrease for the first 9 minutes (until the fourth and fifth 3 minute cycle), however the initial rate on this control run is less than the 0.02 O.D. units per minute desired for the reduction of Cytochrome C. For this experiment the Xanthine oxidase for the standard SOD assays needs to be slightly increased over the amount used.

G. Results using Sigma SOD to establish a standard curve in the classical assay.

This is an indirect quantitative SOD determination in which the indicating scavenger (Cytochrome C) is reacting with the superoxide anion (generated by the xanthine-xanthine oxidase) and the SOD present causes the superoxide anion to be dismutated before it can react with the indicator. The percent inhibition of the control rate is a function of the amount of SOD present. Using standard amounts of SOD from stocks obtained from Sigma, the data in Figure 9 was obtained. The log dose of SOD plotted against probit response avoids the statistical bias introduced by percent data and also will reveal the presence of interfering reactants (reference 4). The 50% inhibition is used to define the ED_{50}, (effective dose for 50% inhibition), which is the defined unit for SOD in this method. The solid line is the least squares fit for experiments run on separate days.

H. Results using Sigma SOD to establish a standard curve using the sensitive assay.

In the sensitive assay the reaction was allowed to proceed for various times to determine the optimum for the burst measurement. The results are given in Figure 10 and showed that 10 minutes could be used for routine assays. The results using Sigma SOD for a standard curve are presented in Figure 11.

I. Results using nitroblue tetrazolium as detector

Because recent workers have used nitroblue tetrazolium (NBT) in their assays (see for instance reference #5), we compared it to cytochrome C. We found no advantages in terms of stability, sensitivity or reproducibility and a distinct disadvantage in the use of the Super Sipper due to the precipitates which formed during the reaction.

VI. REFERENCES

1. McCord, J. M. and I. Fridovich (1969). Superoxide Dismutase, an Enzymic Function for Erythrocuprein. J. Biol. Chem. 244: 6049.

2. Kirby, T. W. and I. Fridovich (1982). A Picomolar Spectrophotometric Assay for Superoxide Dismutase. Anal. Biochem. 127: 435.

3. Crapo, J. D., J. M. McCord, and I. Fridovich (1978). Preparation and Assay of Superoxide dismutase. Methods in Enzymology, 53: 382.

4. Eldred, G. E. and J. R. Hoffert (1981). A Test for Endogenous Interferences in Superoxide Dismutase Assays. Anal. Biochem. 110: 137.

5. Paciorek, J. A. (1985). Human Erythrocyte Superoxide Dismutase Activity During Deep Diving. Eur. J. Appl. Physiol. 54: 163.

PERKIN-ELMER
LAMBDA 7 UV/VIS SPECTROPHOTOMETER
DATE 86-02-18 13:38
SAMPLE ID XANTHINE - XOD
OPERATOR FISHER

METHOD		SCAN/MANUAL
Ø1	ORDINATE MODE	ABS
Ø2	SLIT	2 NM
Ø3	SCAN SPEED	6Ø NM/MIN
Ø4	RESPONSE	Ø.5 S
Ø5	LAMP	332.8 NM
Ø6	CYCLES/TIME	5/ 3.00 MIN
Ø7	PEAK THRESHOLD	Ø.Ø2 A
Ø8	RECORDER	OVERLAY/ AUTO DASH
Ø9	ORD MIN/MAX	Ø.ØØØ / Ø.4ØØ
1Ø	ABSC MIN/MAX	2ØØ.Ø / 32Ø.Ø
11	MEMORIZE /FILES83/	/ /
12	ABSCISSA FORMAT	2Ø NM/CM
13	PRINTER /SCALE/	/

FIGURE 1. Settings on the CRT of page 1 METHOD for the scan of the superoxide generator.

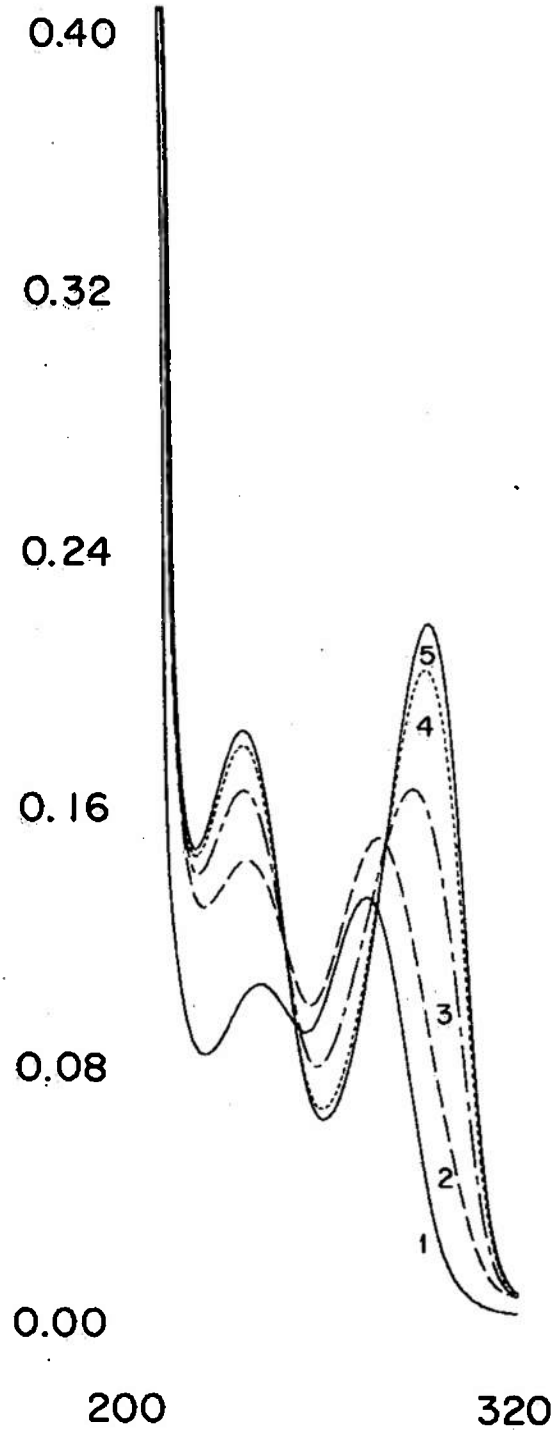

FIGURE 2. Scans of the conversion of xanthine to uric acid by xanthine oxidase.

PERKIN-ELMER

LAMBDA 7 UV/VIS SPECTROPHOTOMETER

DATE 86-Ø2-18 13:3Ø

METHOD SCAN/ MANUAL

SAMPLE	CYCLE	ABSCISSA	ORDINATE
	13:23	272.3 NM	Ø.133 A
	Ø2	276.Ø NM	Ø.152 A
	Ø3	286.2 NM	Ø.167 A
		234.1 NM	Ø.166 A
	Ø4	289.8 NM	Ø.2Ø4 A
		233.6 NM	Ø.18Ø A
	Ø5	290.Ø NM	Ø.218 A
		233.5 NM	Ø.184 A

FIGURE 3. Data page of results from the scans in Figure 2.

PERKIN-ELMER
LAMBDA 7 UV/VIS SPECTROPHOTOMETER
DATE 86-Ø2-19 11:57
SAMPLE ID CYTO C.
OPERATOR FISHER

METHOD		SCAN/MANUAL
Ø1	ORDINATE MODE	ABS
Ø2	SLIT	2 NM
Ø3	SCAN SPEED	6Ø NM/MIN
Ø4	RESPONSE	Ø.5 S
Ø5	LAMP	VIS
Ø6	CYCLES/TIME	5 / 3.ØØ MIN
Ø7	PEAK THRESHOLD	Ø.Ø2 A
Ø8	RECORDER	OFF
Ø9	ORD MIN/MAX	Ø.ØØØ / Ø.2ØØ
1Ø	ABSC MIN/MAX	5ØØ.Ø / 6ØØ.Ø
11	MEMORIZE /FILES83/	/ /
12	ABSCISSA FORMAT	2Ø NM/CM
13	PRINTER /SCALE/	/

FIGURE 4. Settings on the CRT of page 1 (METHOD) for the scan of the cytochrome C detector.

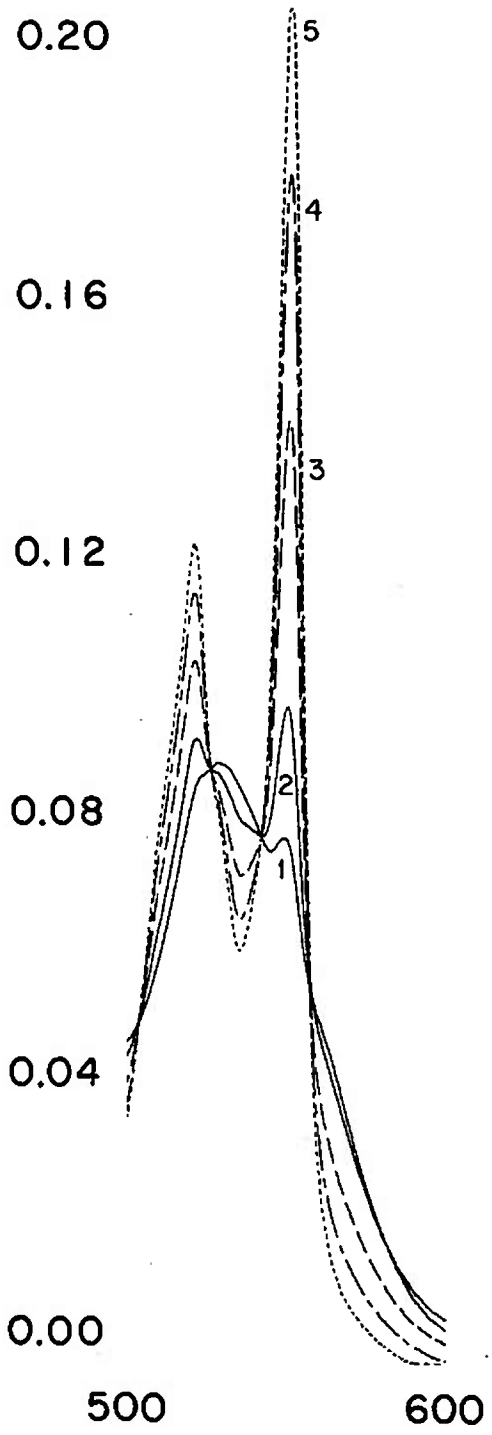

FIGURE 5. Scans of the reduction of cytochrome C by the superoxide generator.

PERKIN-ELMER

LAMBDA 7 UV/VIS SPECTROPHOTOMETER

DATE 86-Ø2-19 11:56

METHOD SCAN/MANUAL

SAMPLE	CYCLE	ABSCISSA	ORDINATE
	11:37	+ 521.9 NM	Ø.Ø87 A
	11:4Ø	+ 549.1 NM	Ø.Ø96 A
	Ø2	+ 549.1 NM	Ø.Ø4Ø6 A
		+ 52Ø.2 NM	Ø.1Ø3 A
	Ø3	+ 549.5 NM	Ø.178 A
		+ 52Ø.2 NM	Ø.113 A
	Ø4	+ 549.4 NM	Ø.2Ø6 A
		+ 52Ø.Ø NM	Ø.121 A
	11:51	+ 549.5 NM	Ø.215 A
		+ 519.8 NM	Ø.124 A
	Ø2	+ 549.4 NM	Ø.Ø232 A
		+ 519.9 NM	Ø.125 A

FIGURE 6. Data page of RESULTS from the scans in Figure 5.

```
PERKIN-ELMER
LAMBDA 7 UV/VIS SPECTROPHOTOMETER
DATE   86-Ø2-19     12:29
SAMPLE ID        STANDARD SOD
OPERATOR      FISHER
WAVELENGTH       55Ø.Ø NM
METHOD                              TIME DRIVE/MANUAL
Ø1  ORDINATE MODE                                 ABS
Ø2  SLIT                                        2 NM
Ø3  RESPONSE                                  Ø.5  S
Ø4  LAMP                                        VIS
Ø5  CYCLES/TIME                   5/        3.00 MIN
Ø6  RECORDER                                    OFF
Ø7  ORD MIN/MAX                   Ø.ØØØ /     Ø.2ØØ
Ø8  CHART SPEED                           1Ø NM/MIN
Ø9  MEMORIZE       /FILES3/        /         /
1Ø  PRINTER             /SCALE/             /
```

FIGURE 7. Settings on the CRT of page 1 (METHOD) for the standard assay.

PERKIN-ELMER

LAMBDA 7 UV/VIS SPECTROPHOTOMETER

DATE 86-Ø2-19 12:29

METHOD TIME DRIVE/MANUAL

SAMPLE	CYCLE	ABSCISSA	ORDINATE
	12:Ø7	55Ø.Ø NM	Ø.Ø38 A
	Ø2		Ø.Ø73 A
	Ø3		Ø.1Ø3 A
	Ø4		Ø.126 A
	Ø5		Ø.139 A
	12:22	55Ø.Ø NM	Ø.144 A
	Ø2		Ø.145 A

FIGURE 8. Results page for the standard assay.

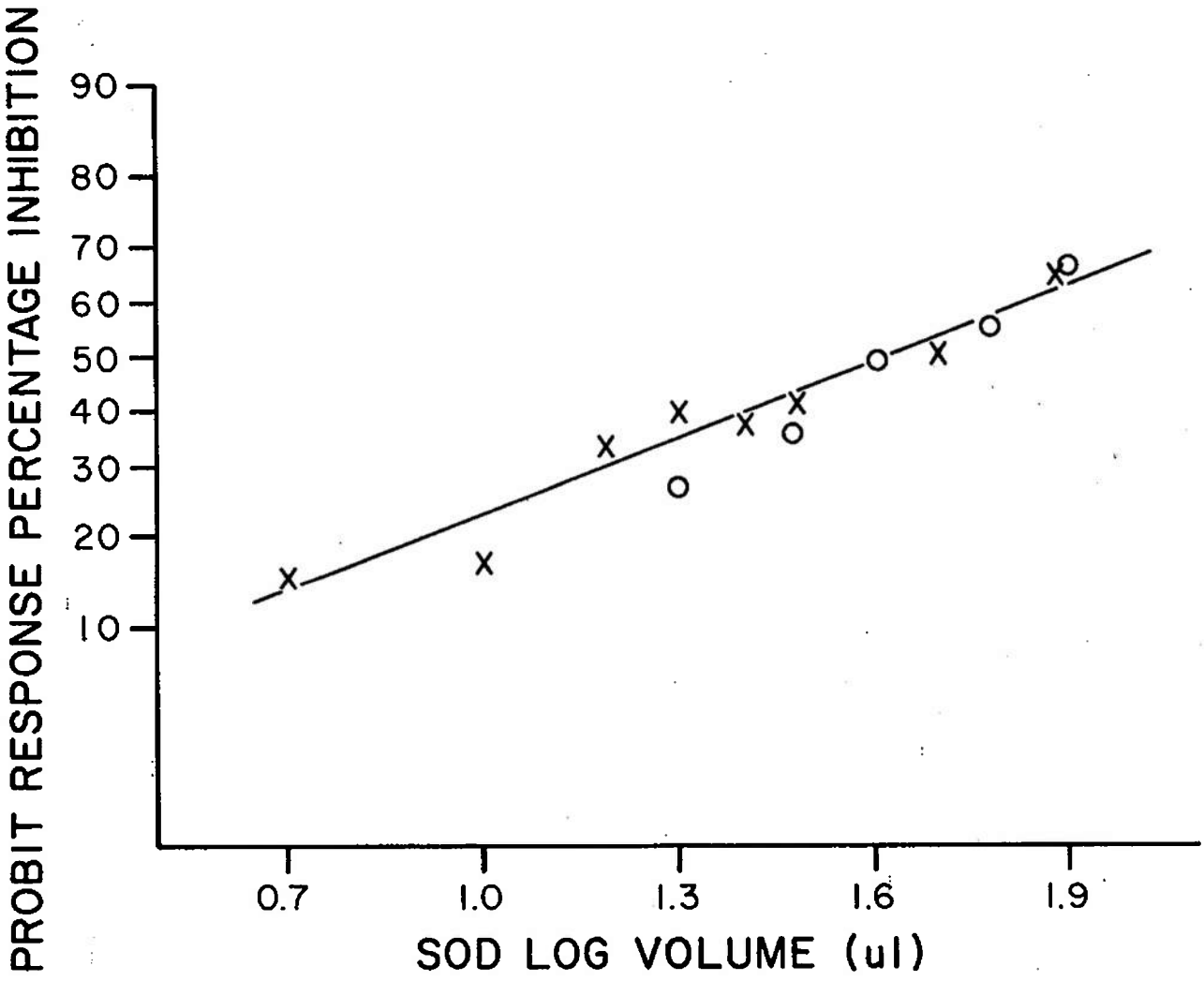

FIGURE 9. The standard curve using Sigma SOD

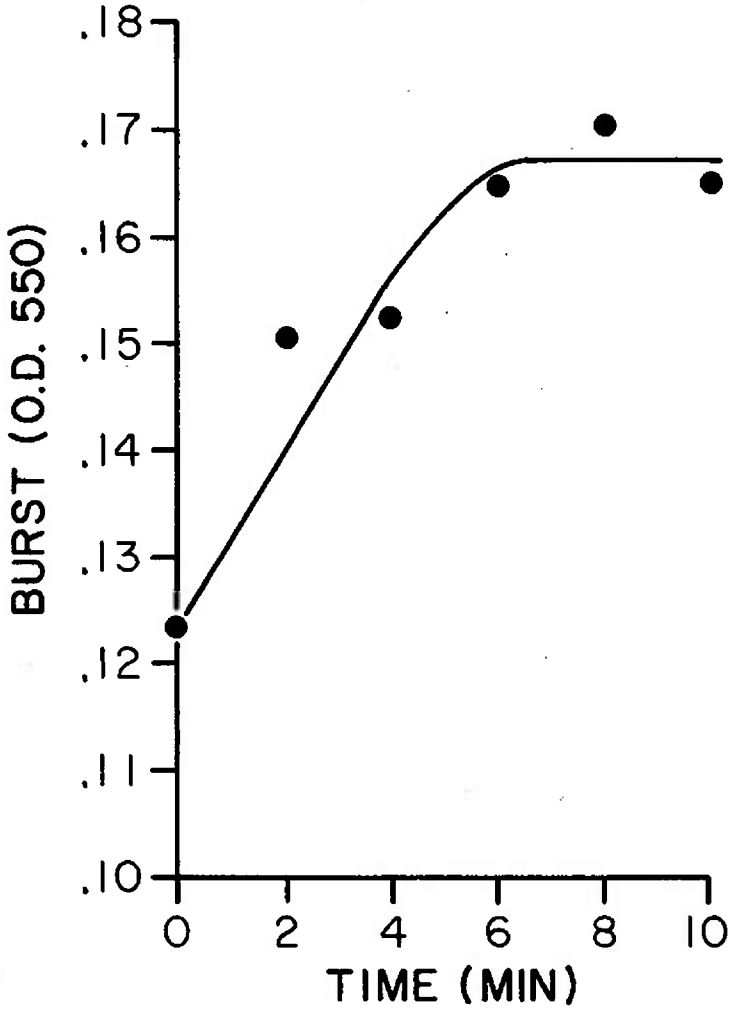

FIGURE 10. Determination of the Optimum time for the Burst measurement

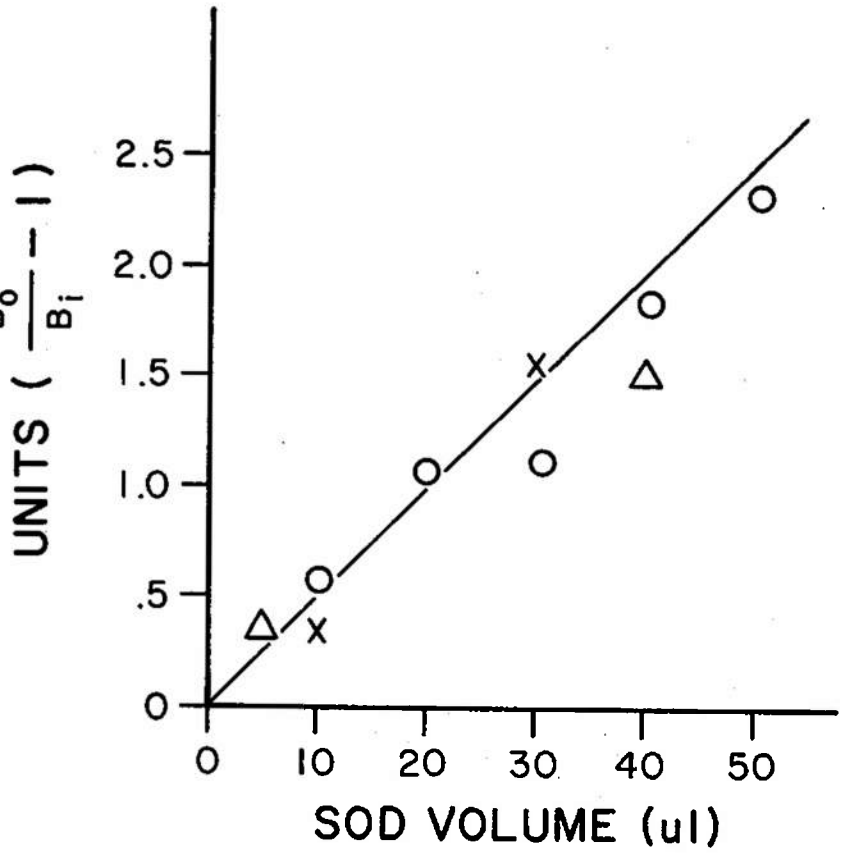

FIGURE 11. Standard curve of the sensitive assay using Sigma SOD. (B_o is the reading at T=10 minutes without SOD and B_i is the reading at 550 nm after adding various volumes of SOD.)

APPENDIX

Reagent Source: Sigma Chemical Co.

1. CYTOCHROME C
   Type VI
   From Horse Heart
   95-100% based on
   Mol. Wt. 12.384
   Approx 10% of the 500 mg
   cytochrome C may be present in the reduced form

2. SUPEROXIDE DISMUTASE
   From Bovine
   Erythrocytes
   Lyophilized powder 75,000 units
   containing 95-98% protein
   (Biuret); balance primarily potassium phosphate
   buffer salts.
   Activity: Approx 3,000 units per mg protein

3. XANTHINE OXIDASE
   (Xanthine oxygen oxidoreductase: EC 1.1.3.22)
   Unit Definition: One unit will convert 1.0 umole
   of oxanthine to uric adic per minute at pH 7.5 at
   25°C.
   Grade III
   From Buttermilk 25 units
   Chromatographically purified
   Suspension in 2.3 M $(NH4)_2SO_4$, 10 mM sodium phosphate
   buffer, pH 2.8, containing 1 mM EDTA and 1 mM sodium
   salicytate
   Activity: 1-2 units per mg protein

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19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Cytochrome C; Superoxide radicals; spectrophotometric assays; cells; diving

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

A number of studies support the idea that superoxide dismutase is one of the enzymes that modulates the threat of oxidative stress. This technical report presents detailed instructions for two methods presently being used at the Naval Submarine Medical Research Laboratory (NSMRL) to assay for superoxide dismutase. Both assays are of the indirect type consisting of two components: a superoxide generator and a superoxide detector. In the first

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method the generator produces the radical at a controlled rate, and in the absence of superoxide dismutase (SOD), the radical produced reacts with the detector. A unit of SOD then has been defined as that amount which will reduce the rate produced by the generator to 50% of its control value. The second method, which is about 100-fold more sensitive, takes advantage of the biphasic nature of the production of the radical and its dismutation both spontaneously and by SOD. By allowing the generator to react for a specific time period before addition of the detector, a burst of reduction is obtained followed by a linear rate after the detector reaches a steady state. The height of this burst in the presence and absence of SOD thereby gives a sensitive assay for which the unit (50% of control burst) is in the picomolar range. These assays are useful in determining SOD in a variety of tissue types such as erythrocytes obtained from Navy divers or cell culture samples that have been exposed to oxidative stress.