A spectrophotometric assay for superoxide dismutase activities in crude tissue fractions

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A sensitive and reliable assay method was developed to characterize crude cell homogenates and subcellular fractions with regard to their superoxide dismutase (SOD) activities. The determination of SOD activities was based on the well-known spectrophotometric assay introduced by McCord & Fridovich [(1969) J. Biol. Chem. **244**, 6049–6055], with partially succinylated (3-carboxypropionylated) rather than native ferricytochrome c as indicating scavenger. Partial succinylation of the cytochrome c resulted in minimization of interference associated with the interaction of cytochrome c with mitochondrial cytochrome c oxidase or cytochrome c reductases. The further increase in specificity, with regard to exclusion of cytochrome c oxidase interference, gained as a consequence of the high pH of 10 enabled the analysis of samples as rich in cytochrome c oxidase activity as the mitochondrial fraction in the presence or absence of membrane-disrupting detergents. Linear relationships for the dependence of the SOD activities with protein concentration were obtained with rat liver homogenate, mitochondrial and microsomal fractions, indicating negligible interference. Furthermore, by choosing a high pH for the assay medium, a 4-fold increase in sensitivity compared with the classical SOD assay, carried out at pH 7.8, was gained as well as a more precise resolution of Cu/Zn-SOD and Mn-SOD by 2 mM-KCN in samples with a high ratio of Mn-SOD to Cu/Zn-SOD, such as mitochondria. The complete trapping of the $O_2^{\cdot-}$ radicals, which was more feasible at pH 10 than at pH 7.8, enabled the application of a simple equation derived for the calculation of appropriately defined units of SOD activity from a single experiment.

INTRODUCTION

Superoxide dismutases and catalase have been detected in a wide variety of mammalian cells (Fridovich, 1983; Chance et al., 1979). These enzymes are thought to play important roles in protecting the cell against the potentially deleterious effects of reactive oxygen species.

Investigations of the subcellular distribution of SODs in rat liver cells have established that they are located in several intracellular compartments (Tyler, 1975; Peeters-Joris et al., 1975). A cyanide-sensitive Cu/Zn-SOD was found to reside in the cytosol and in the inter-membrane space of mitochondria, and a comparatively small portion of the total activity of SOD, attributable to a cyanide-insensitive Mn-SOD, was reported to be restricted to the mitochondrial matrix space.

Previously we have presented evidence for the firm association of significant amounts of SOD as well as catalase with the rat liver microsomal fraction prepared according to standard procedures (Kuthan et al., 1984). Unawareness of SOD activities accompanying the liver microsomal fraction led to misleading interpretations of studies performed in vitro with the use of exogenous SOD as a probe for the involvement of $O_2^{\cdot-}$ radicals in microsomal reactions, such as lipid peroxidation, metabolic activation and redox-cycling of various xenobiotics. It appears necessary, therefore, to define clearly the subcellular fractions used in metabolic studies concerned with reactive oxygen species with respect to their endogenous SOD and catalase content.

Among the tests available for the quantification of SOD activities in biological samples the cytochrome c-based spectrophotometric assay introduced by McCord & Fridovich (1969) enjoys widespread use. In the standard procedure the xanthine/xanthine oxidase system and native cytochrome c serve as the $O_2^{\cdot-}$ source and indicating scavenger respectively. SOD is assayed by its capacity to lower the rate of $O_2^{\cdot-}$-mediated reduction of ferricytochrome c.

In order to eliminate interference by cytochrome c oxidases and peroxidases, low concentrations of cyanide (10–50 μM) as an inhibitor have commonly been used (Salin et al., 1978; Geller & Winge, 1983). This procedure seems to be less satisfactory, however, for the analysis of samples with a relatively high cytochrome c oxidase content, such as mitochondrial fractions.

Alternatively, activities towards cytochrome c of both cytochrome c oxidases and cytochrome c reductases are reportedly virtually eliminated by either acetylation or succinylation (3-carboxypropionylation) of the haemo-protein (Takemori et al., 1962; Azzi et al., 1975; Kuthan et al., 1982).

In the present paper we describe a reliable test suited for the quantification of SOD activities in homogenate, mitochondrial and microsomal fractions, with the use of partially succinylated ferricytochrome c as the indicating scavenger. The experimental analysis of the essential features of this assay is supported by a straightforward theoretical treatment. Furthermore, the advantages and limitations of the more common indirect SOD tests, with special emphasis on cytochrome c-based assays, for the analysis of crude tissue fractions are discussed.

EXPERIMENTAL

Materials

Cytochrome c (bovine heart; type VI), superoxide dismutase (EC 1.15.1.1) from bovine blood and xanthine were purchased from Sigma Chemical Co., Munich, Germany; xanthine oxidase (EC 1.2.3.2) was obtained from Boehringer–Mannheim, Mannheim, Germany; KCN and Triton X-100 were products of Merck, Darmstadt, Germany.

Methods

Livers of 24 h-starved male Sprague–Dawley rats were homogenized in cold 0.25 M-sucrose in 5 mM-EDTA/ 50 mM-Tris/HCl buffer, pH 7.5. Microsomal fractions were prepared by differential centrifugation as described previously (Werringloer & Estabrook, 1975). Mitochondrial fractions prepared from livers of normally fed male Sprague–Dawley rats according to standard procedures (Klingenberg & Rottenberg, 1977) were kindly provided by Dr. G. Blaich, Tübingen, Germany. Microsomal and mitochondrial fractions were kept at 0 °C as concentrated suspensions (about 30 mg/ml and 60 mg/ml respectively) in 0.25 M-sucrose buffered at pH 7.5, and were used within 24 h and 4 h respectively.

Protein was determined by the biuret method (Gornall et al., 1949). Partial succinylation of cytochrome c was performed as described previously (Kuthan et al., 1982). SOD activities were assayed by their capacity to compete with native or partially succinylated ferricytochrome c for $O_2{}^{\cdot-}$ radicals generated by the xanthine/xanthine oxidase system (McCord & Fridovich, 1969; Kuthan et al., 1984). The concentration of bovine Cu/Zn-SOD giving rise to half-maximal inhibition of the $O_2{}^{\cdot-}$-mediated reduction of native ferricytochrome c under standard conditions was assumed to be 3.1 nM (McCord & Fridovich, 1969). The reduction of ferricytochrome c was monitored with a ZWS II spectrophotometer (Sigma K.G., Berlin, Germany) by using the wavelength pair 550 nm minus 557 nm. Calculations of the cytochrome c concentrations were based on an absorption coefficient of 21 mM$^{-1}\cdot$cm^{-1} (Van Gelder & Slater, 1962).

RESULTS

Comparison of native and partially succinylated ferricytochrome c as indicating scavengers in SOD assays

The degree of inhibition of the $O_2{}^{\cdot-}$-mediated reduction of ferricytochrome c by SOD under defined conditions of pH, ionic strength, temperature etc. is, generally, dependent on the concentrations of SOD, ferricytochrome c and $O_2{}^{\cdot-}$ as well as the bimolecular rate constants of the reaction of $O_2{}^{\cdot-}$ with itself, SOD and ferricytochrome c [cf. eqn. (1) in the Appendix]. At saturating concentrations of ferricytochrome c, i.e. complete trapping of the $O_2{}^{\cdot-}$ radicals in the absence of SOD, a simple linear relationship can be obtained for the SOD concentration and the ratio of the rates of reduction of ferricytochrome c determined in the presence and in the absence of SOD [cf. eqn. (10) in the Appendix].

Partial succinylation of cytochrome c has previously been found to change its physicochemical properties dramatically, but with retention of its reducibility by $O_2{}^{\cdot-}$ (Kuthan et al., 1982). Therefore the sensitivity to inhibition by Cu/Zn-SOD of the reduction of native and partially succinylated ferricytochrome c by $O_2{}^{\cdot-}$ was
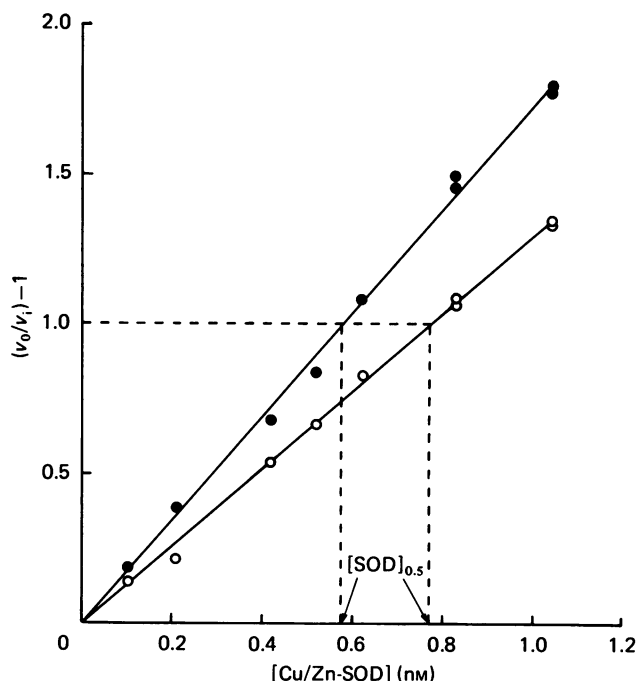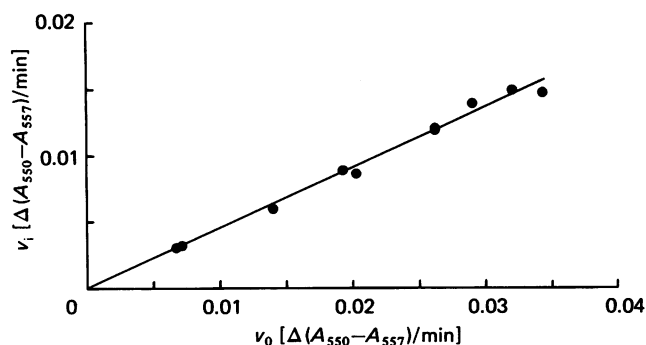

Fig. 1. Inhibition by Cu/Zn-SOD of the $O_2{}^{\cdot-}$-mediated reduction of native (●) and partially succinylated (○) ferricytochrome c

Initial rates of $O_2{}^{\cdot-}$-mediated reduction of native and partially succinylated ferricytochrome c were determined in the absence (v_0) and in the presence (v_i) of the concentrations of Cu/Zn-SOD indicated. The reaction medium contained either 10 μM native ferricytochrome c or 30 μM succinylated ferricytochrome c, 0.1 mM-xanthine and 0.1 mM-EDTA in 50 mM-sodium carbonate buffer, pH 10. The reactions were initiated by adding xanthine oxidase adjusted to give rates of reduction equivalent to 0.025 $\Delta(A_{550}-A_{557})$/min at 25 °C in a total volume of 1 ml. Straight lines were calculated by least-squares analysis.

studied (Fig. 1). To enable a direct comparison with the classical standard assay (McCord & Fridovich, 1969) the concentrations of xanthine oxidase used in these experiments were adjusted to give rates of reduction of both native and succinylated ferricytochrome c of 0.025 $\Delta(A_{550}-A_{557})$/min.

From the linear plots shown in Fig. 1 [SOD]$_{0.5}$ values (i.e. concentrations of SOD that inhibit the rate of $O_2{}^{\cdot-}$-mediated reduction of ferricytochrome c by 50% under specified conditions) of 0.58 nM and 0.77 nM can be derived for the assay with native and succinylated ferricytochrome c respectively. A comparison of these values with the [SOD]$_{0.5}$ value of 3.1 nM determined for the standard assay at pH 7.8 (McCord & Fridovich, 1969) reveals improved sensitivity of about 4-fold and 5.4-fold for the assays carried out at a high pH with modified and native cytochrome c respectively. Thus concentrations of Cu/Zn-SOD down to 0.1 nM can be assayed reliably.

The slightly lower sensitivity determined in the assay with the modified ferricytochrome c as indicating scavenger may, at least in part, reflect the 3-fold higher concentration of this cytochrome as compared with the native cytochrome c.

Fig. 2. Relationship between the initial rates of the $O_2^{\cdot-}$-mediated reduction of partially succinylated ferricytochrome c in the absence (v_0) and in the presence (v_i) of Cu/Zn-SOD

Initial rates of the $O_2^{\cdot-}$-mediated reduction of partially succinylated ferricytochrome c were determined in the absence and in the presence of 1 nM-Cu/Zn-SOD respectively. The concentration of xanthine oxidase was varied appropriately so as to generate the indicated basal rates of reduction (v_0) of the modified cytochrome c. Otherwise the incubation mixture and reaction conditions were as indicated in Fig. 1 legend.

However, the relatively high concentration of the partially succinylated ferricytochrome c, although lowering the sensitivity of the test, proved indispensable for the complete trapping of the enzymically generated $O_2^{\cdot-}$ radicals. As a result of the saturating concentrations of the $O_2^{\cdot-}$-trapping agent and wholly in line with the theoretical prediction [cf. eqns. (9) and (10) in the Appendix], the plots shown in Fig. 1 demonstrate linear relationships between the ratio (v_0/v_i) of the rates of ferricytochrome c reductions as determined in the absence (v_0) and in the presence (v_i) of SOD and the concentration of this enzyme. This allows the simple experimental determination of appropriately defined units of SOD activity (cf. the Appendix):

$$\text{SOD units} = (v_0/v_i) - 1$$

Moreover, the ratio (v_0/v_i) of the initial rates of the $O_2^{\cdot-}$-mediated reduction of ferricytochrome c should be independent of the $O_2^{\cdot-}$ flux in a wide range limited only by the saturation condition.

This conclusion has been supported experimentally for the initial rates of reduction of succinylated ferricytochrome c (v_0) monitored at 550 nm minus 557 nm ranging from about 0.007 to 0.03 $\Delta(A_{550}-A_{557})$/min and with 1 nM-Cu/Zn-SOD for the determination of the corresponding SOD-inhibited rates (v_i) (Fig. 2).

Elimination of interfering cytochrome c oxidase and cytochrome c reductase activities

Since cytochrome c does not specifically react with $O_2^{\cdot-}$ radicals, changes in the rate of reduction, observed upon addition of crude cell homogenates or fractions, may be due to small interfering compounds as well as cytochrome c oxidase and cytochrome c reductase activities.

Low concentrations of cyanide (10–50 μM) are commonly used to eliminate interference by cytochrome c oxidase and cytochrome c peroxidase activities present in cell homogenates and subcellular fractions. This approach may be applied successfully if the pH of the assay medium is considerably less than 10, since at high pH cyanide

concentrations as low as 10 μM significantly inhibit Cu/Zn-SOD (Rotilio et al., 1972). Furthermore, low concentrations of cyanide proved to be unable to suppress the activity of cytochrome c oxidase at pH 10.

Alternatively, partial succinylation of cytochrome c has been demonstrated to diminish cytochrome c oxidase activities contaminating the rat liver microsomal fraction by about 99% at pH 7.6 (Kuthan et al., 1982). Although the residual activity has been found to be negligible when assaying microsomal fractions for their SOD content, the attenuation of the cytochrome c oxidase activity by approx. 30-fold, obtained by raising the pH from 7.8 to 10, would be advantageous, particularly in the analysis of samples exhibiting high cytochrome c oxidase activities, such as the mitochondrial fractions and crude tissue homogenates. In addition, low cytochrome c reductase activities, probably related to the oxidation of endogenous substrates, were observed on incubation of native cytochrome c at pH 10 with the mitochondrial fraction prepared from liver of normally fed rats. These activities were also found to be significant in the presence of 0.2% (v/v) Triton X-100, but were negligible if the native cytochrome c was replaced by its partially succinylated derivative. The latter finding is consistent with the decrease in succinate–cytochrome c_1 reductase activity of about 99% established at pH 7.8 in the absence of detergent for the modified cytochrome c as compared with the native cytochrome c (results not shown).

Resolution of mitochondrial Cu/Zn-SOD and Mn-SOD activities

The presence of both Cu/Zn-SOD and Mn-SOD in rat liver mitochondria has been established (Peeters-Joris et al., 1975; Tyler, 1975). High concentrations of cyanide (1–2 mM) were reported to inhibit Cu/Zn-SOD by 97–99% at pH 10, but were less effective at pH 7.8 (for review see Ysebaert-Vanneste & Vanneste, 1980). In contrast, Mn-SOD is not subject to inhibition by cyanide. Consequently, the discrimination of both types of SOD has been based on their differing sensitivity towards cyanide inhibition (Weisinger & Fridovich, 1973). The dependence of the total and cyanide-insensitive activity of SOD on the concentration of the mitochondrial protein is illustrated in Fig. 3. The linearity of the protein concentration–activity relationships confirms the absence of significant interfering cytochrome c oxidase and cytochrome c reductase activities in the determination of mitochondrial SOD activities. Similar results were obtained if the detergent, employed to disrupt the mitochondrial membranes, was omitted.

The proportion of the Cu/Zn-SOD present in the mitochondrial fraction can be obtained as the difference between the total activity and the activity measured in the presence of 2 mM-KCN (Fig. 3). It represents about 48% of the total activity determined at pH 10 and corresponds to 14.7 pmol/mg of mitochondrial protein. Assuming that the SOD activity measured in the presence of 2 mM-KCN is due to the action of Mn-SOD, the activity of this enzyme at pH 7.8 can be calculated as being equivalent to about 145 pmol/mg of mitochondrial protein. This estimation takes into account that rat liver Mn-SOD shows a decrease in intrinsic activity by 9.14-fold on raising the pH from 7.8 to 10 (Salin et al., 1978). In contrast, mammalian Cu/Zn-SOD displays optimal activity over a wide pH range (Rotilio et al., 1972). To ensure precise quantitative resolution of Cu/Zn-SOD
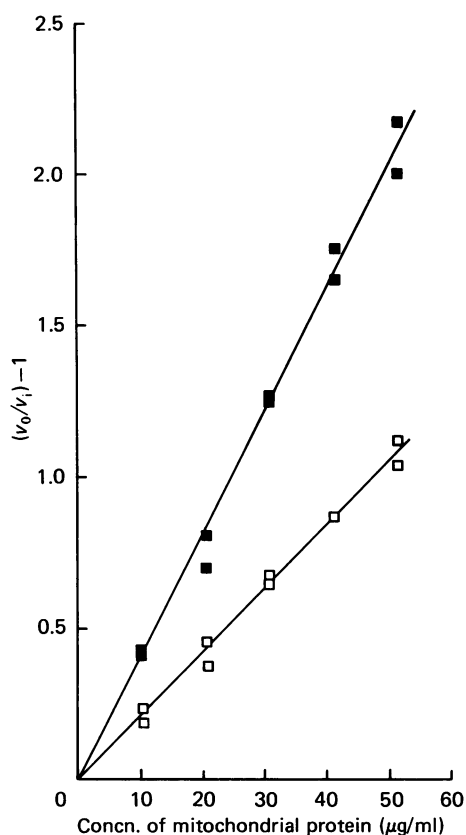
Fig. 3. Total activity (■) and cyanide-insensitive activity (□) of SOD in rat liver mitochondria

Initial rates of $O_2{}^{\cdot-}$-mediated reduction of partially succinylated ferricytochrome c were measured in the absence (v_0) and in the presence (v_i) of the concentrations of mitochondrial protein indicated. The composition of the reaction mixture and the reaction conditions were as described in Fig. 1 legend, with the exception of the additional presence of 0.2% (v/v) Triton X-100.

certain preparations of cytochrome c (McCord et al., 1977). We were unable to substantiate this presumption, however, as incubation with diethyldithiocarbamate for 90 min at 37 °C, which was found to inactivate Cu/Zn-SOD completely (Heikkila & Cohen, 1977), did not cancel the apparent cyanide-triggered increase in the rate of ferricytochrome c reduction. Rather, this spectral change might be attributable to the formation of cytochrome c–cyanide complexes (Lemberg & Barrett, 1973).

Quantitative determination of SOD activities in rat liver homogenate and the microsomal fraction

SOD activities in crude liver homogenate and microsomal fractions, expressed as equivalents of bovine Cu/Zn-SOD, are summarized in Table 1. The amount of 146 pmol of SOD/mg of protein of liver homogenate, corresponding to about 20 nmol of SOD/g wet wt. of liver, tallies well with the value reported by Lindmark & Müller (1974).

Previous determinations of SOD activities at pH values below 8 (Tyler, 1975; Peeters-Joris et al., 1975) indicated that about 90–95% of the total SOD in rat liver homogenate should be attributable to cyanide-sensitive Cu/Zn-SOD. Since Mn-SOD displays a decrease in activity in rat liver homogenate by about 9.1-fold at pH 10 compared with pH 7.8 (Salin et al., 1978), the apparent absence of this enzyme activity, as suggested by complete inhibition of SOD activities at pH 10 by 2 mm-KCN (Table 1), seems reasonable. Although the resolution of Cu/Zn-SOD and Mn-SOD by using cyanide as an inhibitor in samples with a high ratio of Mn-SOD to Cu/Zn-SOD such as mitochondria is greatly facilitated at pH 10, Mn-SOD may be less easy to detect at this high pH in samples with a high ratio of Cu/Zn-SOD to Mn-SOD.

Relatively high cyanide-sensitive SOD activities have been found to be associated with the rat liver microsomal fraction (Table 1). These activities could not be removed by extensive washing with either 0.3 m-sodium pyrophosphate buffer, pH 7.6, containing 0.25 m-sucrose, or 0.15 m-KCl.

Furthermore, SOD activities, in contrast with catalase activities linked to the microsomal fraction, displayed no latency phenomenon in the presence of 0.2% (v/v) Triton X-100 (Kuthan et al., 1984).

So far, we have no explanation for the firm association of Cu/Zn-SOD with the microsomal fraction, but it gives

and Mn-SOD, appropriate corrections were made to account for the apparent stimulation (about 20%) in the rates of the $O_2{}^{\cdot-}$-mediated reduction to cytochrome c to be reproducible after addition of 2 mm-KCN at pH 10 regardless of the use of partially succinylated or native cytochrome c. This cyanide-related effect was assumed to be due to a contamination by SOD encountered with

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Table 1. SOD contents in rat liver homogenates and microsomal fractions

SOD contents are expressed as equivalents of bovine Cu/Zn-SOD (determined at pH 10), and are given as means ±S.D. for the numbers of preparations indicated in parentheses. Where indicated, measurements were made in the presence of 0.2% (v/v) Triton X-100.

	SOD content (pmol/mg of protein)		
	Homogenate	Microsomal fraction	
	Triton present	Triton absent	Triton present
Total content	146 ± 22 (4)	9.6 ± 0.9 (7)	11.7 ± 1.2 (4)
CN⁻-sensitive fraction of total content	148 ± 22 (4)	9.0 ± 0.9 (7)	11.8 ± 1.2 (4)

a clue as to the variety of contradictory or misleading interpretations of experiments performed with purified SOD as a probe for the involvement of $O_2{}^{\cdot-}$ radicals in microsomal reactions.

DISCUSSION

For the analysis of SOD activities a variety of indirect tests, which have been classified as positive and negative assays (Misra & Fridovich, 1977), have been developed.

Positive spectrophotometric assays for SOD, based on the inhibition by $O_2{}^{\cdot-}$ of the horseradish-peroxidase-catalysed peroxidation of dianisidine which is relieved by SOD, were proposed by Misra & Fridovich (1977). Application of these assays in the analysis of crude cell homogenates or fractions requires, however, the complete elimination of catalase activities in these samples before assay.

Tests of the negative type are based in general on the inhibition by SOD of an $O_2{}^{\cdot-}$-mediated oxidation or reduction of any of a number of compounds. Xanthine oxidase acting on xanthine or hypoxanthine has widely been used as a continuous source of $O_2{}^{\cdot-}$ radicals. Indicating scavengers for $O_2{}^{\cdot-}$ include, e.g., ferricytochrome c and Nitro Blue Tetrazolium (McCord & Fridovich, 1969; Beauchamp & Fridovich, 1971). Ferricytochrome c is univalently reduced by $O_2{}^{\cdot-}$ to ferrocytochrome c, whereas Nitro Blue Tetrazolium is primarily reduced to the free radical, producing the insoluble reduced formazan. In addition, tests based on the (aut)oxidation of adrenaline (epinephrine), pyrogallol, 6-hydroxydopamine (2,4,6-trihydroxyphenethylamine), hydroxylamine or sulphite, initiated or propagated by the $O_2{}^{\cdot-}$ radical, have been put forward (for reviews see Misra & Fridovich, 1977; Flohé & Ötting, 1984).

These free-radical chain reactions, as well as the above-mentioned peroxidation of dianisidine, lack, however, straightforward reaction pathways. This makes them less useful for the analysis of crude biological samples containing redox-active compounds such as thiols, quinols and peroxides, which may interfere unpredictably with the initiation, propagation or termination of free-radical reactions.

In contrast, the univalent reduction of native or partially succinylated ferricytochrome c proceeds in a pure second-order reaction (Koppenol et al., 1976; Kuthan et al., 1982). In the meantime several modifications of the cytochrome c-based classical negative assay method of McCord & Fridovich (1969) have been put forward. These modifications were usually aimed at further increasing the sensitivity of the original test beyond the nanomolar range of SOD concentrations (Kirby & Fridovich, 1982; Salin & McCord, 1974).

The modified cytochrome c-based assay described in the present paper meets the need for a sensitive SOD test applicable in the analysis of crude biological samples such as mitochondrial and microsomal fractions. The turbidity associated with these suspensions has been accounted for by adopting the dual-wavelength spectrophotometric approach. One major disadvantage of SOD tests based on native cytochrome c with regard to their usefulness in the analysis of crude tissue fractions, namely potential interference by cytochrome c oxidase or cytochrome c reductase activities, has been shown to have been eliminated by partial succinylation of the haemoprotein.

Possible non-enzymic interferences by small redox-active compounds in biological samples with the modified cytochrome c may be tested for by monitoring the changes in the extent of reduction of the haemoprotein in the presence of excess exogenous SOD added subsequently to the sample being investigated. If enzymic interference as well as non-enzymic interference in the assay have been found to be negligible, the changes in the rate of $O_2{}^{\cdot-}$-mediated reduction of the cytochrome c derivative are considered to reflect the activity of SOD present in the sample. The validity of this assumption should, however, be checked. High concentrations of cyanide specifically reverse the changes in $O_2{}^{\cdot-}$-mediated cytochrome c reduction provoked by Cu/Zn-SOD, whereas preincubation of cellular fractions with 2% (w/v) SDS has been reported to inactivate selectively the Mn-SOD from rat liver (Geller & Winge, 1983).

To conclude, the SOD test developed, based on partially succinylated cytochrome c, has been found to be sensitive and reliable and should be particularly useful for the analysis of crude suspensions of homogenate or cellular fractions.

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APPENDIX

THEORETICAL TREATMENT OF THE QUANTITATIVE DETERMINATION OF SOD

In the presence of SOD the initial rate of reduction of ferricytochrome c (cyt^{3+}) by $O_2^{\cdot-}$ radicals is given by:

$$v_i = \frac{V_{max.}[cyt^{3+}]}{(k_1'/k_3)[O_2^{\cdot-}] + (k_2/k_3)[SOD] + [cyt^{3+}]} \quad (1)$$

where $V_{max.}$ represents the steady-state rate of $O_2^{\cdot-}$ production, and k_1', k_2 and k_3 denote the second-order rate constants for the reaction of $O_2^{\cdot-}$ with itself, SOD and ferricytochrome c respectively (Kuthan et al., 1982).

In the absence of SOD eqn. (1) simplifies to:

$$v_0 = \frac{V_{max.}[cyt^{3+}]}{(k_1'/k_3)[O_2^{\cdot-}] + [cyt^{3+}]} \quad (2)$$

Division of eqn. (2) by eqn. (1) and rearrangement yields:

$$(v_0/v_i) - 1 = \frac{(k_2/k_3)[SOD]}{(k_1'/k_3)[O_2^{\cdot-}] + [cyt^{3+}]} \quad (3)$$

Since 1 unit of SOD activity is defined as the concentration of SOD ($[SOD]_{0.5}$) that inhibits the reduction of ferricytochrome c by 50%, i.e. $v_0/v_i = 2$ (McCord et al., 1977), the following equation can be derived from eqn. (3):

$$1 \text{ unit} = [SOD]_{0.5} = \frac{(k_1'/k_3)[O_2^{\cdot-}]_{0.5} + [cyt^{3+}]}{(k_2/k_3)} \quad (4)$$

The term 'units' of SOD may therefore be defined as follows:

$$\text{units} = \frac{[SOD]}{[SOD]_{0.5}} \quad (5)$$

By using eqn. (5) the following expression is derived from eqn. (4):

$$[SOD] = \frac{(k_1'/k_3)[O_2^{\cdot-}]_{0.5} + [cyt^{3+}]}{(k_2/k_3)} \times \text{units} \quad (6)$$

Insertion of eqn. (6) into eqn. (3) results in:

$$(v_0/v_i) - 1 = \frac{(k_1'/k_3)[O_2^{\cdot-}]_{0.5} + [cyt^{3+}]}{(k_1'/k_3)[O_2^{\cdot-}] + cyt^{3+}]} \times \text{units} \quad (7)$$

Transformation of eqn. (7) yields:

$$\text{units} = \frac{(k_1'/k_3)[O_2^{\cdot-}] + [cyt^{3+}]}{(k_1'/k_3)[O_2^{\cdot-}]_{0.5} + [cyt^{3+}]} \times [(v_0/v_i) - 1] \quad (8)$$

At saturating concentrations of ferricytochrome c, i.e. complete trapping of the $O_2^{\cdot-}$ radicals generated in the absence of SOD, simple linear functions for the calculation of SOD in terms of activity units or concentration are obtained:

$$\text{units} = (v_0/v_i) - 1 \quad (9)$$

or

$$[SOD] = [(v_0/v_i) - 1][SOD]_{0.5} \quad (10)$$

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