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A Modified Spectrophotometric Assay of Superoxide Dismutase

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A simple and rapid method for the assay of superoxide dismutase in biological samples is described. Present method takes advantage of the inhibition of NADH-dependent-nitroblue tetrazolium reduction by the dismutase. Inhibition of the chromogen formation by superoxide dismutase was linear with increase in enzyme concentrations. The chromogen extract in butanol was stable even up to 48 hr. Superoxide dismutase activity, as assayed by the modified method, was in good agreement with that obtained using other methods.

Superoxide dismutase (EC1.15.1.1) has been recognised to play an important role in body defense mechanisms against the deleterious effects of oxygen free radicals in biological systems[1,2]. Its significance in various types of pathogenic response, especially toxic chemical injury to cells[3], makes the study of the functional status of superoxide dismutase isozymes[4] an important aspect of research in biomedical and environmental studies[5]. Such studies, where a large number of samples are used, require a simple, sensitive and rapid assay method for superoxide dismutase. Available methods based on xanthine oxidase coupling[6], autooxidation of epinephrine[7], and riboflavin[8], pulse radiolysis[9], and NADH-phenazine methosulphate-nitroblue tetrazolium formazan inhibition[10] for the assay of superoxide dismutase are generally found cumbersome and time consuming. In view of this, the formazan inhibition method[10] was modified in which formazan formed at the end of the reaction was extracted into butanol layer upon inactivation of the reaction with acetic acid, before spectrophotometric assay. The modified method proved to be useful in handling a large number of samples simultaneously with ease for clinical purposes. The details are presented in this communication.

Purified preparations of superoxide dismutase from bovine blood containing 2700 units/mg protein were procured from Sigma Chemical Co., USA. Also, Cu-Zn superoxide dismutase from lung was partially purified by 90% ammonium sulfate salting out of the lung cytosol fraction. Pooled lungs of rats (150-160 g body wt, from Industrial Toxicology Research Centre Animal Colony) were homogenised in 0.25 *M* sucrose and differentially centrifuged under cold conditions to get cytosol fraction for this purpose. Ammonium sulphate-fractionated superoxide dismutase preparation was dialysed overnight against 0.0025 *M* Tris-HCl buffer (*p*H 7.4) before use for the enzyme assay.

The assay system for superoxide dismutase was adopted from the method of Nishikimi *et al.*[10] and modified in the following fashion. Assay mixture contained 1.2 ml sodium pyrophosphate buffer (*p*H 8.3, 0.052 *M*), 0.1 ml 186 μM phenazine methosulphate, 0.3 ml 300 μM nitroblue tetrazolium, 0.2 ml NADH (780 μM), appropriately dilute enzyme preparation and water in a total volume of 3 ml. Reaction was started by the addition of NADH. After incubation at 30°C for 90 sec, unless otherwise specified, the reaction was stopped by the addition of 1.0 ml glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4.0 ml of *n*-butanol. The mixture was allowed to stand for 10 min, centrifuged and butanol layer was taken out. Colour intensity of the chromogen in the butanol was measured at 560 nm in Unicam SP 500 spectrophotometer against butanol. A system devoid of enzymes served as control. The spectrum of the chromogen solution in acetic acid (without extraction) and after extraction in butanol of control as well as enzyme-treated mixture was also taken in a Cary 219 Double Beam Recording Spectrophotometer.

For comparative purposes, superoxide dismutase activity was also assayed according to the original method of Nishikimi *et al.*[10] and McCord and Fridovich[6].

One unit of the enzyme activity is defined as enzyme concentration required to inhibit the optical density at 560 nm of chromogen production by 50% in one min under the assay conditions, and expressed as specific activity in milliunits/mg protein. When the assay was done for 90 sec, the factor 2/3 was applied for calculating units.

Phenazine methosulphate, NADH, nitroblue tetrazolium and other biochemicals were procured

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Table 1—Superoxide Dismutase Activity as Assayed by Modified Method and by the Methods of Nishikimi *et al.*[10] and McCord & Fridovich[6].

[Values are average ± standard deviation of five determinations in each case. Values given in parentheses represent specific activity expressed in terms of protein content in the assay system.]

Enzyme (ml)	Superoxide dismutase activity (m units/min in the assay system)		
	Modified method	Nishikimi *et al.*[10]	McCord & Fridovich[6]
*Pure enzyme**			
0.05 ml	588 ± 5 ($3.26 \times 10^{+6}$)	634 ± 70 ($3.52 \times 10^{+6}$)	532 ± 60 ($2.95 \times 10^{+6}$)
0.10 ml	976 ± 15 ($2.71 \times 10^{+6}$)	980 ± 48 ($2.72 \times 10^{+6}$)	945 ± 70 ($2.62 \times 10^{+6}$)
0.20 ml	1437 ± 26 ($1.99 \times 10^{+6}$)	1464 ± 50 ($2.03 \times 10^{+6}$)	1408 ± 90 ($1.95 \times 10^{+6}$)
0.30 ml	1830 ± 0 ($1.69 \times 10^{+6}$)	1707 ± 37 ($1.58 \times 10^{+6}$)	1780 ± 95 ($1.64 \times 10^{+6}$)
Purified enzyme from lung† 0.05 ml	1744 ± 20 ($1.93 \times 10^{+5}$)	1643 ± 65 ($1.82 \times 10^{+5}$)	1690 ± 80 ($1.87 \times 10^{+5}$)

*Pure superoxide dismutase (Sigma make) diluted 50-fold and used as indicated.

†Partially purified superoxide dismutase preparation of rat lung cytosol containing 0.18 mg/ml of the preparation.

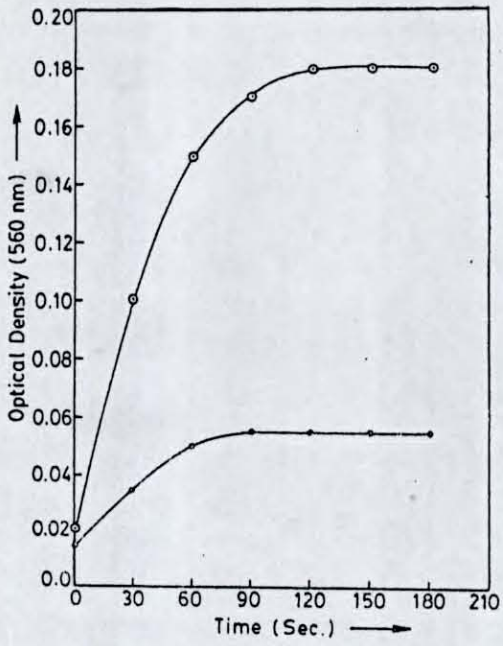

Fig 1—Time course of the NADH-phenazine methosulphate-nitroblue tetrazolium formazan formation. [Control, (○—○); superoxide dismutase, (●—●). Reaction was stopped at varying time intervals with acetic acid and formazan formed was extracted in the butanol layer. Assay system and extraction method are described in the text.]

from Sigma Chemical Co., USA. Other chemicals used were either BDH Analar or equivalent.

Table 1 shows the activity of pure superoxide dismutase preparation as well as the lung superoxide dismutase sample assayed by different methods. When the activity obtained with varying amounts of enzyme was compared, proportionality was observed by all the three methods tried. Statistically also the extraction method gave data comparable to other methods. In all the cases the activity obtained by the modified method is approximately similar to those assayed by the other two methods. The chromogen content of the system with and without extraction with butanol was similar. Apart from the similarities in the enzyme activity by different methods, the failure of a second butanol extraction to extract any further chromogen in the modified method indicates that the quantitative extraction of the chromogen occur in the very first step of the extraction under the assay conditions. The extracted colour was stable up to 48 hr. Also, with varying concentrations of 50-fold diluted superoxide dismutase preparation, proportionality between amount of enzyme and activity was apparent. Data recorded in Fig. 1 for the time course of the control and superoxide dismutase treated reaction indicate that in 90 sec the reaction was optimal. Eventhough, the optical density values are low, they can be scaled up to higher range by using increasing amounts of the reactants. The low level of optical denisity at 0 time is due to the low amounts of reactants in the reaction mixture. Since the chromogen production in the absence of superoxide dismutase reaches maximum at 90 sec due to the complete interaction of reactants, there was no further change in optical density even in the presence of superoxide dismutase after 90 sec. In order to follow initial rate, we have taken 50% inhibition of 1 min optical density change as activity.

Acetic acid in the concentration used was found to arrest the formazan formation. Also, when acetic acid was added prior to NADH addition, reaction did not initiate.

The visible spectra of the chromogen with and without extraction into the butanol layer against corresponding controls were similar with only one peak at 560 nm. The corresponding spectra after incubation with 0.05 ml of 50-fold diluted pure superoxide dismutase (Sigma) also were similar. The spectra in either case were identical in pattern, indicating that optical density determined at λ_{max}, i.e. at 560 nm, is valid for the assay in the modified method also. Further, the spectral characteristics were unaffected by the acetic acid treatment and butanol extraction of chromogen.

Activity obtained by the modified method and the original method for superoxide dismutase in various fractions obtained during purification of lung

superoxide dismutase by ion-exchange chromatography also tallied. With precise timing of NADH addition and acetic acid, a large number of samples can be conveniently assayed in a single stretch, saving considerable time as compared to the individual direct spectrophotometric assay. Since a large number of samples in series can be extracted one after another, there is no loss of time also. Triplicate runs of the same samples showed less than 5% variation, thus reproducibility of the results is better with the modified method than with the direct spectrophotometric assay. In a fast reaction like superoxide dismutase where controlling of time, sequence of additions of reactants and mixing are important, the proposed test tube method for the assay of superoxide dismutase will be simpler than the direct reading of the reaction on the cuvettes. Since superoxide dismutase is generally assayed in the partially purified preparations, the addition of acetic acid instead of trichloro acetic acid to stop the reaction helps to dissolve the protein so that a subsequent centrifugation step can be avoided. The butanol extract of chromogen was also free from turbidity. Thus by incorporating the procedure of chromogen extraction, as done in the case of a few dehydrogenases, the NADH-phenazinemethosulphate-nitroblue tetrazolium formazan inhibition reaction can be adopted for the rapid, simple, sensitive and reliable assay of a large number of samples for superoxide dismutase activity.

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