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Superoxide dismutase determination by oxygen electrode

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A simple and rapid method for the assay of superoxide dismutase in biological samples is described. The method depends upon the generation of $O_2^-\cdot$ in the reaction mixture due to the photoreduction of riboflavin in the presence of EDTA and the subsequent O_2 uptake from the medium. The O_2 uptake was measured with the help of an oxygen electrode. The inhibition of O_2 uptake due to the dismutation of $O_2^-\cdot$ in the presence of tissue homogenate was used to measure the activity of superoxide dismutase.

THE superoxide radical $O_2^-\cdot$, intermediate in the reduction of molecular oxygen, is directly or indirectly involved in many reactions of biomolecules, specially lipids and proteins. Superoxide anion is formed by the one-electron reduction of oxygen in the erythrocytes and in the tissues[1]. The generation of $O_2^-\cdot$ often causes injury to the biological system. The enzyme superoxide dismutase (SOD) has been detected in many tissues and is known to protect the cell from damage by the highly reactive superoxide free radical[2-4]. SOD catalyses the disproportionation of the superoxide radical according to the following reaction:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 .$$

The substrate – evidently, the superoxide radical – used for the assay of SOD has to be generated enzymatically or nonenzymatically in the assay medium, which contains an easily measurable indicator reacting with $O_2^-\cdot$ (ref. 5). All analytical methods for the determination of SOD are based on this ability to accelerate the dismutation of $O_2^-\cdot$ and thereby require a source of $O_2^-\cdot$ and a system for detecting it. All methods are indirect except the polarographic method and suffer from the inherent disadvantage of nonlinear response to increasing enzyme concentration[5]. The routinely used procedure for the assay of SOD employs detection of $O_2^-\cdot$ by measuring the reduction of cytochrome C (ref. 1), nitroblue-tetrazolium (NBT)[6] or epinephrine[7].

In the present method, the $O_2^-\cdot$ radical served as the substrate for SOD, generated nonenzymatically in a light-dependent reaction with the concomitant uptake of oxygen from the test medium. Riboflavin can be photochemically reduced in the presence of an oxidizable substance. Upon reoxidation in air, reduced flavins will generate $O_2^-\cdot$ and the superoxide radical in turn dismutates to oxygen in the presence of SOD, resulting in the inhibition of oxygen uptake. In the absence of light, riboflavin is not reduced and no uptake of O_2 is observed. In the earlier methods the generation of superoxide radicals was used to reduce the colourless NBT to a blue formazon[6] or riboflavin-sensitized photooxidation of dianisidine was augmented by SOD[8]. As flavins undergo light-dependent reaction in the presence of an electron donor, with subsequent generation of $O_2^-\cdot$ due to the photoreduction of O_2 (ref. 8), a method based on this property has been designed to determine the activity of SOD in tissue homogenate with the help of an oxygen electrode.

O_2 measurement was done by the Hansatech oxygen electrode (UK) connected to a suitable *X–Y* recorder. The electrode was calibrated for maximum oxygen concentration as its upper limit by air-saturated distilled water at room temperature. The response at minimum oxygen tension was checked by 2% solution of sodium dithionate. The calibration of the electrode was checked during the course of the experiment. The reaction chamber was illuminated by an incandescent lamp providing light at an intensity of 1500 $\mu E\ m^{-2}\ s^{-1}$. The concentration of O_2 at any given time was evaluated by the method of tangents using the *X–Y* plot. Evaluation of the *X–Y* plot by this method provides rapid inspection of trace amounts of O_2 in the steady state. The inhibition of O_2 uptake in the presence of SOD was obtained by comparing the slope with that of the blank and was used as a measure of the activity of SOD.

Male wistar strain rats weighing 150–180 g were housed in polypropylene cages with free access to drinking water and basal diet. The animals were sacrificed by decapitation and their livers removed, washed in 1 M phosphate buffer (pH 7.4) and soaked with filter paper. The tissue was weighed and a 10% homogenate was prepared in 0.25 M sucrose solution. The homogenate was centrifuged at $30,000 \times g$ for 30 min to obtain a crude extract. Partial purification of SOD was done by centrifuging the crude extract at $100,000 \times g$ for 1 h in Sorvall RC 28S centrifuge, followed by ammonium sulphate precipitation of the supernatant. SOD from liver

was partially purified by 90% ammonium sulphate precipitation and salting out the cytosolic fraction. Ammonium-sulphate-fractionated SOD precipitation was dialysed overnight with changes against 1 mM Tris–HCl buffer (pH 7.4) and was used in the enzyme assay. Protein content was estimated by the method of Lowry *et al.*[9].

The reaction mixture consisted of 0.15 mM riboflavin, 0.5 µM KCl and 0.1 mM EDTA in a total volume of 3 ml of 1 M phosphate buffer (pH 7.4). All additions were made directly into the well of the reaction chamber. The reaction was started by illuminating the reaction mixture for 2.5 min. The partially purified enzyme (0.05–0.2 ml) was injected into the reaction chamber after the system had stabilized and the recorder showed steady oxygen uptake. Each of the experiment was performed in twofold concentration range of the enzyme. A blank run, consisting of adding the boiled enzyme to the reaction mixture, was performed with each test sample. The O_2 uptake at any given interval of time was expressed as µmol h^{-1} mg^{-1} protein. One unit of enzyme was defined as the concentration of the enzyme required for 50% inhibition of O_2 uptake.

Figure 1 represents the uptake of O_2 in the presence of partially purified enzyme. Sodium azide (0.5 mM) completely blocked the O_2 uptake as it quenches the $O_2^-\cdot$ radical and showed no uptake of O_2. This indicates that no oxygen species other than $O_2^-\cdot$ is generated in the reaction mixture. Cyanide in the reaction mixture was used to inhibit the catalase activity. The activity of SOD was studied by the dismutation of $O_2^-\cdot$ and the proportionate inhibition in the O_2 uptake was recorded. The $O_2^-\cdot$ radicals were generated in the reaction mixture by the photoreduction of riboflavin in the presence of EDTA. The inhibition in the O_2 uptake was calculated as a measure of SOD activity.

Riboflavin in the presence of an electron donor generates $O_2^-\cdot$ due to the photoreduction of O_2. The uptake of O_2 was recorded in the presence of varying concentration of riboflavin. Riboflavin up to 0.15 µm showed a linear increase in O_2 uptake (Figure 2).

The presence of 0.1 mM EDTA in the reaction mixture increased the uptake of O_2. The effect was observed in blank as well as in the test, as shown in Table 1. In

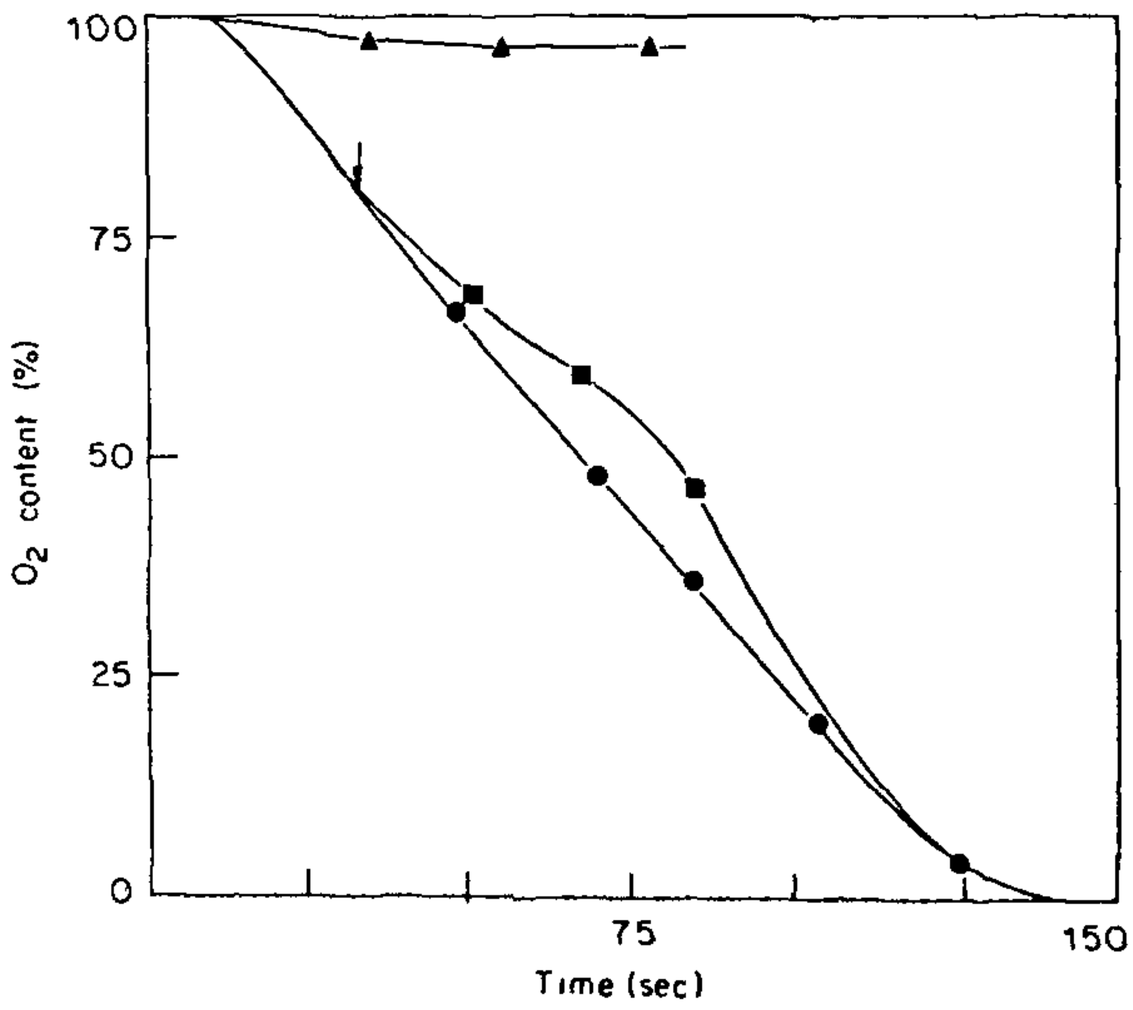

Figure 1. The uptake of O_2 as recorded by the oxygen electrode Symbols (●-●) and (■-■) represent the uptake of O_2 in blank and after the addition of partially purified enzyme, respectively The time of addition of the enzyme is marked by an arrow. Symbol (▲-▲) represent the uptake of O_2 in the presence of sodium azide.

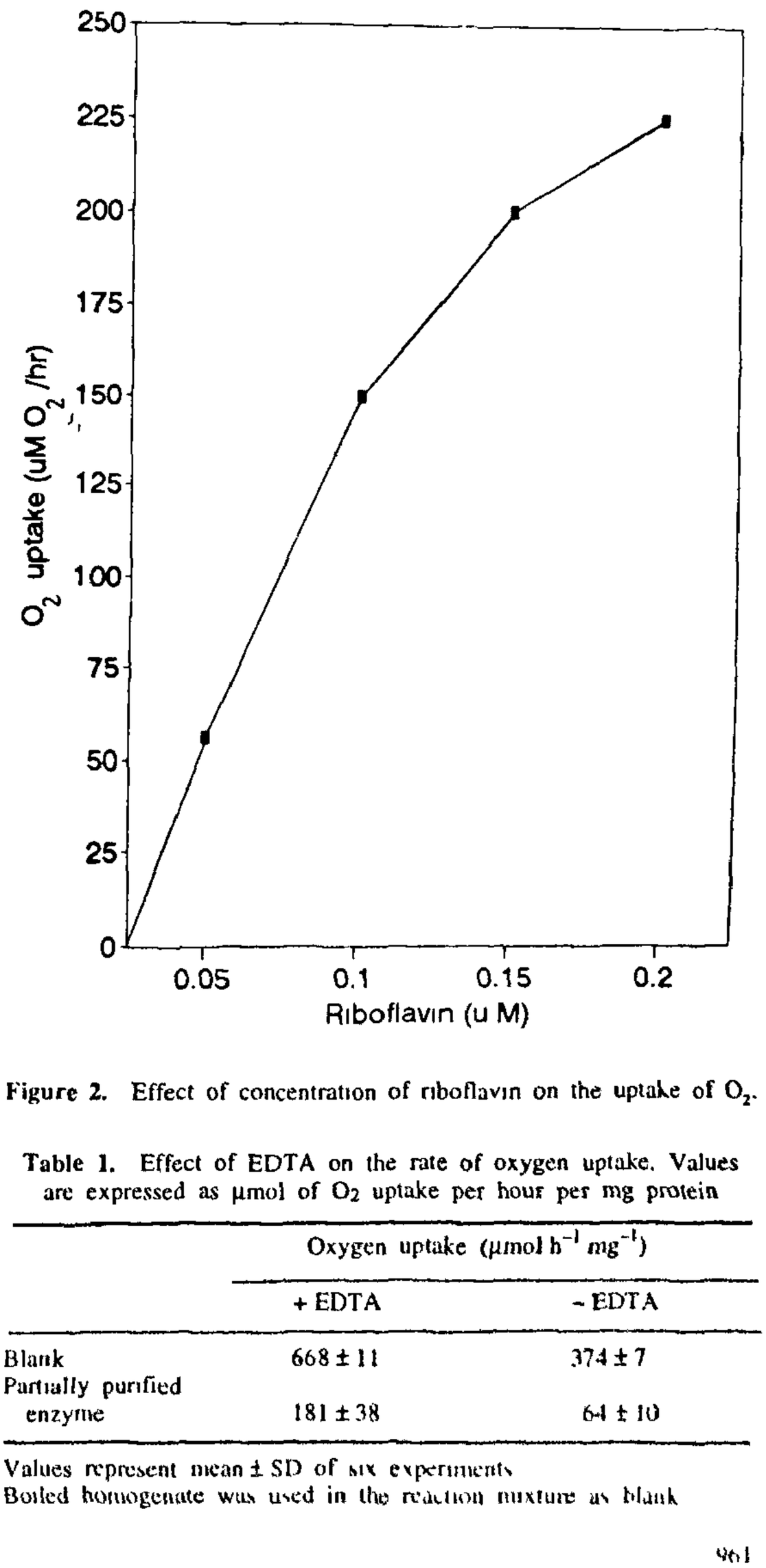

Figure 2. Effect of concentration of riboflavin on the uptake of O_2.

Table 1. Effect of EDTA on the rate of oxygen uptake. Values are expressed as µmol of O_2 uptake per hour per mg protein

	Oxygen uptake (µmol h^{-1} mg^{-1})	
	+ EDTA	- EDTA
Blank	668 ± 11	374 ± 7
Partially purified enzyme	181 ± 38	64 ± 10

Values represent mean ± SD of six experiments
Boiled homogenate was used in the reaction mixture as blank

a photosensitized reaction, excited riboflavin in the presence of EDTA generated $O_2^-\cdot$. The production of $O_2^-\cdot$ in the medium was made stable by EDTA. This also favours metastable $O_2^-\cdot$ to gain stability in an electron-rich medium[11].

The O_2 uptake was measured in the presence of partially purified enzyme. The percentage inhibition of O_2 uptake is represented in Figure 3. Inhibition of 50–70% of O_2 uptake should be observed with the addition of the enzyme.

The addition of ferric chloride or cupric acetate (1 mM) to the reaction mixture showed no effect on the uptake of O_2 in blank or in the presence of the enzyme (Table 2).

The riboflavin-sensitized photoreduction and its augmentation by SOD was influenced by pH. The enzyme activity was high at pH 7.4 and a sharp pH optima was observed. The inhibition of O_2 uptake was measured at different pH values. The maximum inhibition of O_2 uptake was at pH 7.4. The percentage inhibition in the presence of enzyme was calculated and converted to units of SOD/mg protein.

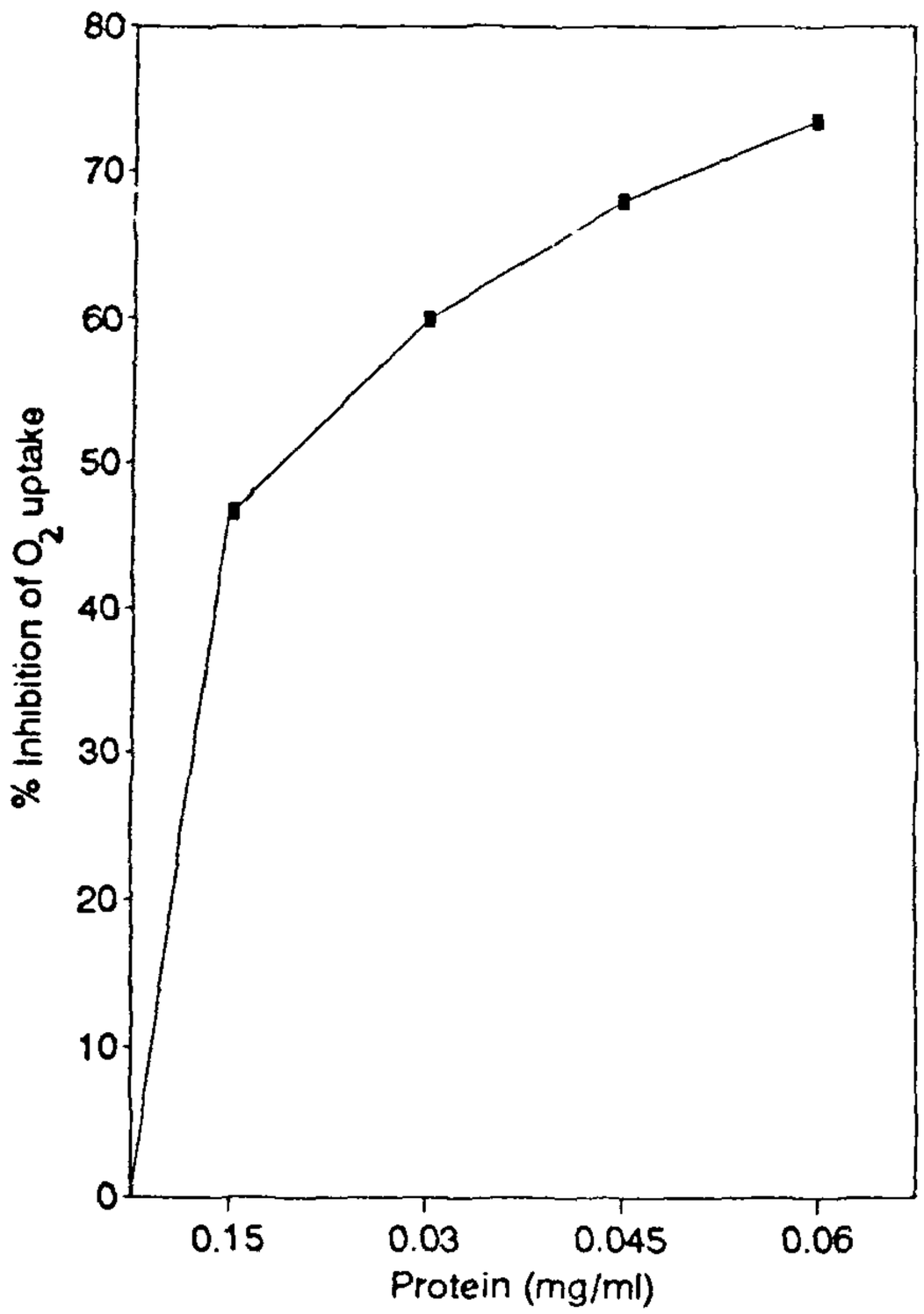

Figure 3. Inhibition of O_2 uptake in the presence of partially purified enzyme.

Table 2. Effect of metal ions on the rate of oxygen uptake. Values are expressed as µmol of O_2 uptake per hour per mg protein

	Oxygen uptake (µmol h^{-1} mg^{-1})		
	No addition	+ Fe^{3+}	+ Cu^{2+}
Blank	671 ± 11	664 ± 10	660 ± 11
Partially purified enzyme	180 ± 35	189 ± 31	192 ± 42

The reaction mixture was as described in the text. Ferric chloride or cupric acetate (1 mM) was added to the reaction mixture and the O_2 uptake was recorded by the O_2 electrode. Values represent mean ± SD of six experiments.

The inhibition of $O_2^-\cdot$ generation in the riboflavin–EDTA medium can be used to assay SOD in a biological sample. The method is simple, rapid and reproducible. The assay is not influenced by the ions present in the homogenate. The absence of interfering reaction, as evident by the assay in its linearity with the protein content in the assay mixture, helps to estimate the activity of SOD in biological fluids. The sensitivity of the oxygen electrode to determine the O_2 uptake and the activity of SOD accurately can, therefore, be used as an assay procedure for kinetic studies.

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