A Comparative ESR Study of Some Paramagnetic Materials as Probes for the Noninvasive Measurement of Dissolved Oxygen in Biological Systems

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The ESR properties of three types of paramagnetic material, active charcoal, fusinite and a stable nitroxide radical 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPONE), were examined in order to evaluate their suitability as probes to measure dissolved intra- and extra-cellular oxygen. Although, with changes in oxygen concentration, a greater change in the linewidth of ESR signals was observed with fusinite or active charcoal, it took a long time (15 min for active charcoal and more than 6 h for fusinite) for equilibrium to be achieved. On the other hand, equilibrium was reached very rapidly in the case of the TEMPONE spectra although the sensitivity to changes in oxygen concentration was only moderate. Furthermore, since lipid bilayers are permeable to TEMPONE, this compound can be used to measure intracellular oxygen concentration when employed in combination with membrane-impermeable spin-broadening reagents which act on ESR signals arising from extracellular probes. A perdeuterated derivative of TEMPONE is useful in that it gives a greater signal-to-noise ratio and greater sensitivity to changes in oxygen concentration. In conclusion, active charcoal is suitable as a probe for extracellular oxygen in a system where changes are slow, while nitroxide is a versatile probe for measuring rapidly changing intra- and extra-cellular oxygen concentrations.

Keywords ESR oximetry; paramagnetic probe; line broadening; fusinite; active charcoal; TEMPONE

Oxygen plays a central role in the energy metabolism of biological systems, acting as a terminal electron-acceptor in the respiratory chain. It acts also as an oxidant in the detoxification of xenobiotics by microsomes. There are also a number of pathological phenomena in which active oxygen species derived from oxygen play a key role. Therefore, accurate measurement of oxygen concentrations is fundamental and a prerequisite for understanding such physiological and pathological phenomena. Thus, there is a real need to measure oxygen concentrations in specific tissues or, if possible, within the cells of those tissues.

Use of electron spin resonance (ESR) to measure oxygen concentrations, named ESR oximetry, has definite advantages over other techniques and appears most promising. The development and potential of ESR oximetry has been adequately reviewed by Swartz and Glockner.[1] This technique is based on the interaction of molecular oxygen with paramagnetic probes which broadens the ESR signals of the probe. It is noninvasive and causes minimal perturbation to biological systems, compared with the conventional Clark electrode technique. The technique can be applied to extracellular or intracellular oxygen in tissues and in whole animals if appropriate probes are used.

The feasibility of ESR oximetry for measuring *in vivo* oxygen concentrations depends principally on the probes. Probes for ESR oximetry should be (1) stable and inert paramagnetic compounds, (2) sensitive to changes in oxygen concentration and (3) the intracellular probes must be incorporated easily into cells. In the present study, we investigated the characteristics of three probes with respect to these properties: the paramagnetic probes were active charcoal, fusinite (a special type of coal), and a nitroxide radical 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPONE) or its perdeuterated derivative TEMPONE-d_{16}.

Materials and Methods

Active charcoal for chromatography was obtained from Wako Pure Chemicals (Osaka, Japan). Gas-permeable Teflon (TPX) film (50 μm thick) was kindly provided by Mitsui Petrochemical Industries (Tokyo, Japan). Fusinite was purchased from Illinois EPR Research Center (Urbana, ILL, U.S.A.). TEMPONE was from Eastman (Rochester, NY, U.S.A.) and TEMPONE-d_{16} was synthesized by the condensation of deuterated ammonia and acetone-d_6 followed by oxidation.[2] The paramagnetic broadening agent, potassium tris(oxalato)chromate(III) trihydrate ((oxalato)chromate) was synthesized according to Bailar and Jones.[3] ESR spectra were measured at room temperature (23 °C) using a JEOL-JES RE-1X X-band spectrometer with 100 kHz field modulation. ESR spectra were recorded at 5 mW of microwave power, 10 μT modulation width, and a 1.25 mT/min field sweep rate unless otherwise stated. Fusinite and active charcoal were finely ground in a mortar made of amber and suspended in 10 mM Hepes-Na buffer (pH 7.40) and the nitroxides were dissolved in the same buffer. Samples of 10 μl were put into a cylindrical tube (1.5—2 mm in diameter) made of TPX film which was then placed in the center of a quartz pipe (5 mm in outer diameter) set in a cylindrical TE_{011} mode cavity. Through the quartz pipe was passed a sufficiently humidified mixture of nitrogen and oxygen gases at the velocity of 200 ml/min.

Results and Discussion

The spectra of an aqueous solution of 0.1 mM TEMPONE and suspensions of active charcoal and fusinite were all sensitive to changes in oxygen concentration of the gas phase in equilibrium with the aqueous medium of the samples. Figure 1 shows the response time of the three types of probe to changes in oxygen concentration: 100% nitrogen gas was applied to the sample which had been equilibrated with air and the change in the peak height of the spectra was monitored as a function of time. Spectra of TEMPONE reached

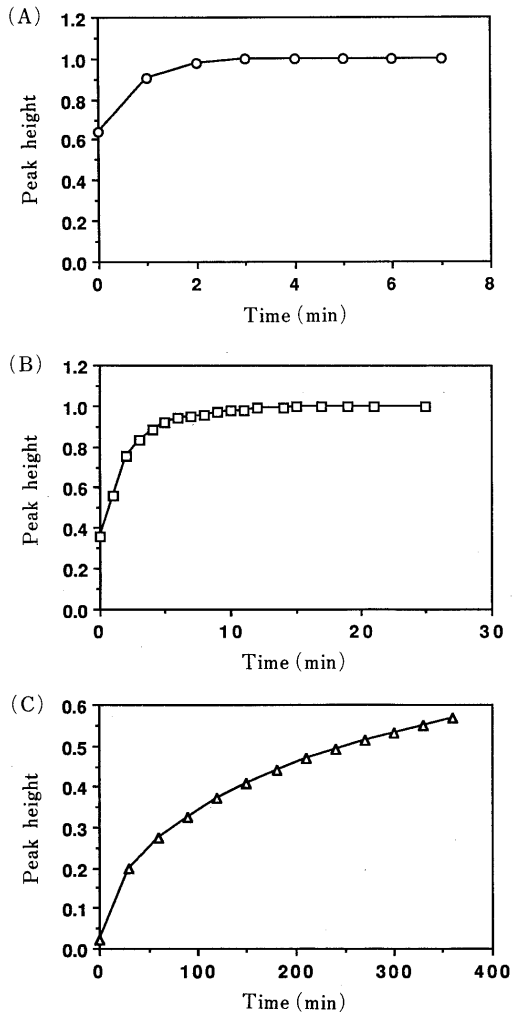

Fig. 1. Change in Peak Height of ESR Spectral Lines with Time After Changing the Atmosphere from Air to 100% Nitrogen

Paramagnetic samples were dissolved or suspended in 10 mM Hepes-Na (pH 7.4). (A) 0.1 mM TEMPO solution. Peak height of the center line of three spectral lines was plotted. (B) Active charcoal suspension. (C) Fusinite suspension.

equilibrium within 3 min of changing the gas composition. This rate is principally determined by the diffusion of oxygen molecules across the TPX film.[4] Spectra of active charcoal took 15 min to reach equilibrium while equilibration was very slow for fusinite, more than 6 h.

On the other hand, the sensitivity of the spectra to changing oxygen concentration was highest for fusinite as shown in Fig. 2A where the spectral linewidths 10 min after the change in oxygen concentration are plotted as a function of the oxygen concentration in the gaseous mixture. Also plotted are the spectral parameters of TEMPONE and active charcoal in equilibrium with the gaseous phase. The sensitivity to changes in oxygen concentration is second highest for active charcoal and poorest for TEMPONE. These results contradict to some extent previous observations: Swartz *et al.*[5] reported that sensitivity was highest for sucrose char and second highest for fusinite. Glockner and Swartz[6] reported that the equilibration time for fusinite was about 2 h. These discrepancy may be due to differences in the origin and physicochemical properties of the probes, including their particle size.

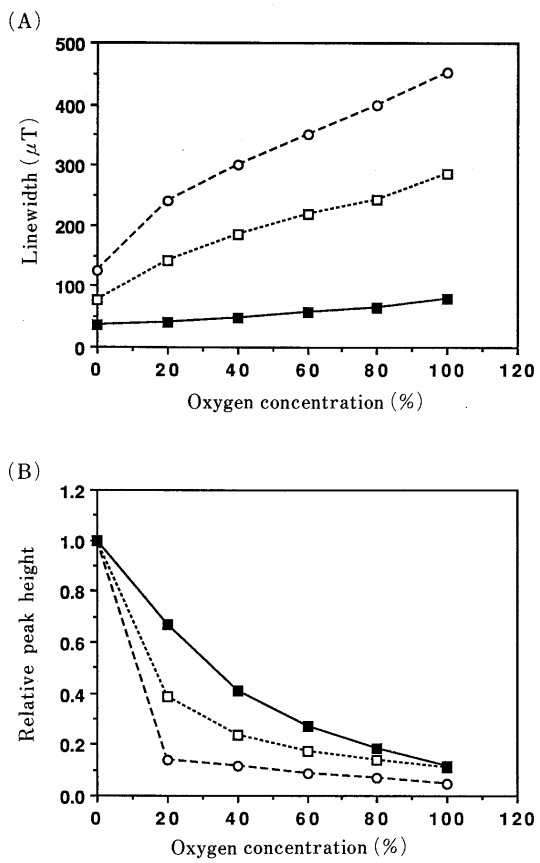

Fig. 2. (A) Linewidth or (B) Peak Height of ESR Spectral Lines as a Function of the Oxygen Concentration in a Mixture of Oxygen and Nitrogen

Spectra of active charcoal (□) and TEMPONE (■) were measured after equilibrium was established while the spectra of fusinite (○) were recorded 10 min after the change in the composition of gaseous mixture.

In Fig. 2B, spectral peak heights are plotted instead of linewidth as a function of oxygen concentration. In this way, the apparent sensitivity of TEMPONE can be increased.

Because of its fast response to changes in oxygen concentration, TEMPONE seems to be the first choice as a probe to be used in biological systems where oxygen concentrations change rapidly, in spite of the fact that its sensitivity to oxygen concentration change is not very great. The response of TEMPONE and TEMPONE-d_{16} was examined in detail over the range 0—20% oxygen concentration (Fig. 3), since this is the range that is physiologically important. The slope of the linewidth-oxygen concentration plot is greater for TEMPONE-d_{16} than for TEMPONE.

Spin-exchange broadening was observed for the spectra of TEMPONE solutions of concentrations higher than 0.1 mM and of TEMPONE-d_{16} solutions of concentrations higher than 0.05 mM. As shown in Fig. 3, linewidth increases with increasing radical concentrations. However, the linewidth of the exchange-broadened spectra is still sensitive to changes in oxygen concentration similar to that of the spectra obtained with dilute TEMPONE solutions. The slopes of the plotted lines obtained with different radical concentrations in Fig. 3 (A) or (B) are similar.

In order to measure intracellular oxygen concentrations,

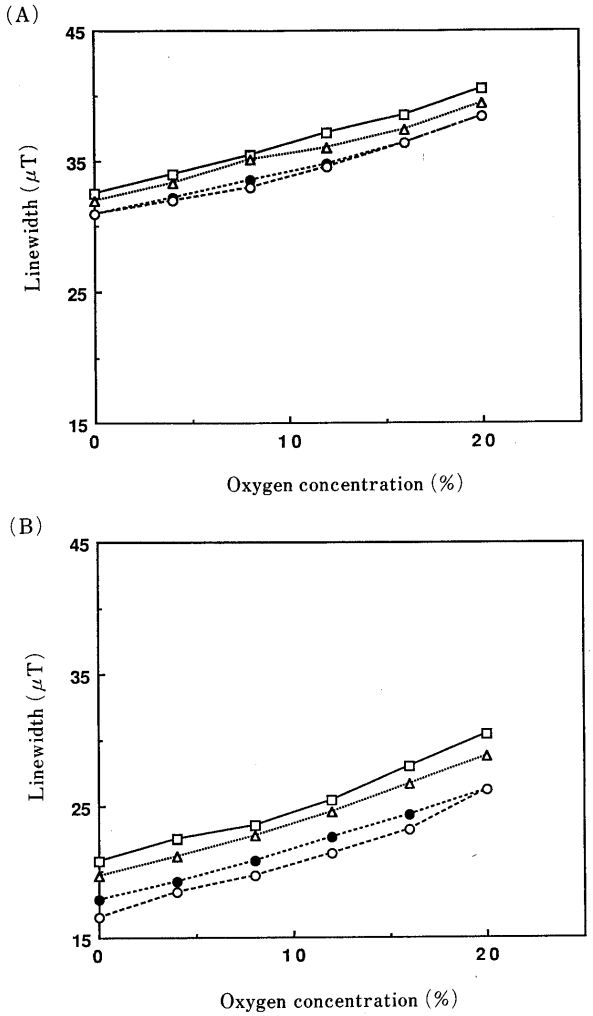

Fig. 3. Linewidth of the Central Line of Spectra of TEMPONE (A) or TEMPONE-d_{16} (B) Solutions in 10 mM Hepes-Na (pH 7.4) as a Function of the Oxygen Concentration in a Mixture of Oxygen and Nitrogen

○, 0.02 mM; ●, 0.1 mM; △, 0.3 mM; □, 0.5 mM.

a probe must reach the intracellular space. This is not easy with solid probes such as active charcoal or fusinite. In contrast, TEMPONE can be easily introduced into the cytoplasm since the plasma membrane is permeable to TEMPONE and analogous neutral nitroxide radicals. Use of TEMPONE in combination with spin broadening agents such as a paramagnetic metal complex, (oxalato) chromate, provides a method to determine intracellular oxygen. Since the membrane-impermeable broadening reagent broadens the ESR spectra of extracellular spin probes, only the spectra of intracellular spin probes can be observed. This method was previously used by us to measure intracellular or intravesicular volume[7] and has also been applied to the measurement of intracellular oxygen concentration.[8,9] However, careful examination of this technique with respect to the effect of the broadening agent on the spectral parameters of intracellular probes has not yet been carried out. In the present study, we have measured the spectral linewidth of TEMPONE inside liposomes as a function of oxygen concentration. Squares in Fig. 4 represent the effect of oxygen on the linewidth of the spectra from intravesicular

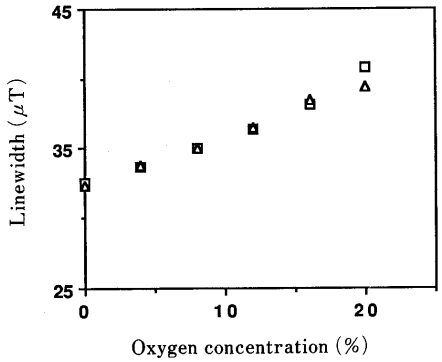

Fig. 4. Linewidth of the Central Line of Spectra of TEMPONE in PBS Plotted Against the Oxygen Concentrations in an Oxygen and Nitrogen Mixture

△, 0.5 mM TEMPONE solution; □, 0.5 mM TEMPONE + liposome (250 mg asolectin/ml) + 55 mM Cr-oxalate.

TEMPONE molecules. Triangles in Fig. 4 shows the dependence of the linewidth on oxygen concentration for TEMPONE molecules in solution. The two results are in good agreement indicating that in fact the extracellular spin broadening agent only broadens the spectra from extravesicular probes without affecting intravesicular probes.

In conclusion, the present study has shown that fusinite is unsuitable for use as a probe for ESR oximetry because its response is very slow although active charcoal may be suitable as a probe for extracellular oxygen in a system where the change is slow. The nitroxide TEMPONE is a versatile probe for the measurement of rapidly changing intra- and extra-cellular oxygen concentrations and TEMPONE-d_{16} is even better, because of its higher signal-to-noise ratio and higher sensitivity to oxygen, compared with TEMPONE. The application of ESR oximetry to mouse tissue *in vivo* using an L-band ESR spectrometer and TEMPONE-d_{16} is in progress in our laboratory at Kyushu University.

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