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ELECTRON SPIN RESONANCE-SPIN TRAPPING. DETECTION OF SUPEROXIDE FORMATION DURING AEROBIC MICROSOMAL REDUCTION OF NITRO-COMPOUNDS.

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Summary. ESR measurements demonstrate that in aerobic systems reduction of aromatic nitro-compounds by rat liver microsomes leads to the production of superoxide which may be detected as its adduct with nitrone spin traps.

Introduction. The ESR-spin trapping technique,[1] by which rective radicals are added to nitroso-compounds or nitrones to give a more stable nitroxide spin adduct, has recently been employed in biological systems.[2,3] The majority of radicals likely to be found in such systems (semiquinones, flavin semiquinones, ascorbate etc.) are thermodynamically rather stable anions, and as such are unlikely to participate in the spin-trapping reaction, but superoxide, $O_2^{\cdot-}$, is an exception to this generalization, as Harbour and Bolton have demonstrated.[4] Superoxide and/or HO_2. reacts with nitrones e.g. 5,5' -dimethyl-1-pyrroline-1-oxide (DMPO), reaction 1, to give a relatively long-lived adduct nitroxide whose ESR spectrum is characteristic of the addend. Direct detection of $O_2^{\cdot-}$ at neutral pH will ordinarily require its generation in high concentration. After immediate freeze-quenching, the sample can then be examined at low temperature. In contrast, the spin-

Abbreviations: ESR - Electron Spin Resonance, DMPO - 5,5' -Dimethyl-1-pyrroline-1-oxide, PBN - Phenyl-N-t-butyl nitrone.

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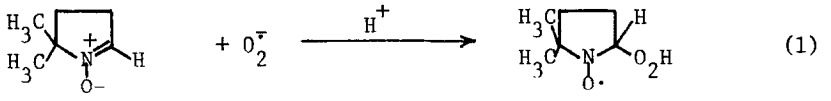
trap method does not require high radical initiation rates and can be carried out at ambient temperatures.



(1)

This method for superoxide detection was applied to a biological system by Harbour and Bolton, who showed[3] that superoxide is generated during chloroplast illumination, but we know of no further such application. We here report the spin-trapping of $O_2^{\cdot-}$ during microsomal reductions.

Mason and Holtzmann have previously shown[5] that incubation of aromatic nitro-compounds ($ArNO_2$) with rat liver microsomes and the co-factor NADPH generates radical anions ($ArNO_2^{\cdot-}$) detectable with ESR spectroscopy. The predominant nitro-reductase in liver microsomes appears to be[6] cytochrome c reductase. In common with the majority of nitroreductases, it is inhibited by oxygen. Indirect evidence has been presented[7] that oxygen inhibition in this system arises through oxidation of the primary radicals by molecular oxygen to produce superoxide. Reactions (2) and (3) indicate the proposed mechanism. Reaction (3) is known[8] to be facile in chemical systems. We have obtained direct confirmation of this mechanism by the spin-trapping technique.

$$ArNO_2 \xrightarrow{\text{enzyme}} ArNO_2^{\cdot-} \qquad (2)$$

$$ArNO_2^{\cdot-} + O_2 \longrightarrow ArNO_2 + O_2^{\cdot-} \qquad (3)$$

Methods. Microsomes were prepared as follows. The livers from young male Sprague-Dawley rats were perfused with 0.15 M NaCl then homogenized in a teflon/glass homogenizer in an ice bath. The homogenate was centrifuged twice at 9000 g for 15 minutes. The supernatant was then centrifuged at 105,000 g for 45 minutes in a Beckman L-265 B ultracentrifuge, the pellet resuspended in a TRIS/sucrose buffer (0.05 M TRIS, pH 7.5, 0.25 M sucrose) and centrifuged again at 105,000 g for 60 minutes. The final pellet was resuspended in TRIS/sucrose buffer and aliquots were frozen at -70 °C (1 ml preparation contained the microsomes from 0.5 g liver).

Radical generation was similar to that described by Mason and Holtzman.[5] Incubation mixtures contained nitro-compound (0.5 - 5 mM) and a NADPH generating system comprising NADP (0.7 mM), glucose-6-phosphate (10 mM) and glucose-6-phosphate dehydrogenase (1.3 units/ml) in phosphate buffer (0.06 M, pH 7.2). Mixtures were used either aerated, depleted in oxygen by the bubbling of nitrogen gas, or saturated with oxygen gas. After addition of

microsomes (0.2 ml/ml of solution) the microsomal system was thoroughly mixed before an aliquot was rapidly transferred to an aqueous flat cell for ESR examination at 25 °C. For spin-trapping experiments either DMPO or phenyl-N-t-butyl nitrone (PBN) was added to the incubation mixture shortly before the addition of microsomes. Final concentrations were 0.08 M. DMPO, a gift from Dr. K. S. Chen, was redistilled before use. PBN was obtained from Aldrich Chemical Company.

ESR measurements were made using X-band Varian E-9 and E-109 spectrometers equipped with 100 kHz field modulation. g-Values and hyperfine splittings were measured relative to a solution of Fremy's salt (g^9 = 2.0055, $a(N)^{10}$ = 13.09 G) contained in a capillary on the flat surface of the aqueous cell.

Results and Discussion. Incubation of $ArNO_2$ (nitrofurantoin (N-(5-nitro-2-furfurylidene)-1-aminohydantoin), nitrofurazone (5-nitro-2-furaldehyde semicarbazone), misonidazole (Ro-07-0582, 1-(2-hydroxy-3-methoxypropyl)-2-nitromidazole) or nitrobenzoate) with the microsomal preparation in dearated solution gave strong ESR-signals from the corresponding nitro-radical anions, the majority of which have been previously reported.[7,11] It also has been reported[5,7] that the radicals from nitrofurantoin and nitrobenzoate cannot be detected if the solution is aerated. We find that in aerated and oxygenated mixtures radical spectra can be observed after an induction period which varies with the nitro-compound used (nitrofurantoin<misonidazole< nitrobenzoate).[12] Following this induction period, a steady state in radical concentration is achieved. The Figure shows the data obtained for nitrofurantoin in oxygenated solution. The steady-state concentration is proportional to the square root of the enzyme concentration, indicated that during the steady state radical termination is kinetically second order, most probably reflecting disproportionation.

The induction period was approximately five times shorter in aerated solutions suggesting that it reflects depletion of molecular oxygen in the medium, i.e. the solution becomes anaerobic owing to the occurrence of reaction (3). The following results confirm this hypothesis.

Inclusion of DMPO in the incubation mixture gave the spectrum of the nitrone adduct with superoxide, with hyperfine splittings and g-value identical to those previously reported.[4] Significantly, the spectrum of the adduct nitroxide was detected only during the induction period. Control experiments

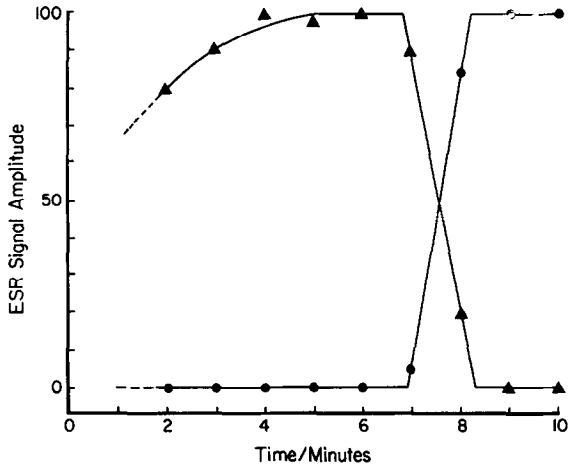
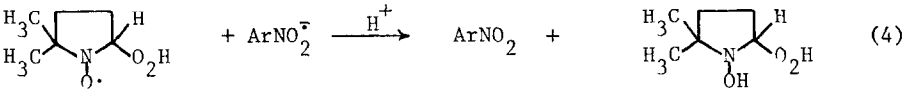


Fig. Changes in free radical ESR signal amplitudes with time following mixing of rat liver microsomes with an oxygen-saturated solution of nitrofurantoin containing DMPO. ▲ DMPO- $O_2^{\bar{\cdot}}$ adduct ● $ArNO_2^{\bar{\cdot}}$. Signal amplitudes are expressed as percentages of the maximum observed for each species.

established that both nitro-compound and enzyme were essential for the generation of the spin adduct. After the induction period we observed the nitro radical anion, as shown in the Figure. We infer the occurrence of reactions(2) and (3). Similar data were obtained using PBN.

The nitro-radical anions were not trapped by the nitrone at an appreciable rate. After their appearance the nitroxide radicals rapidly disappeared. We attribute this to disproportionation as in (4).



(4)

There is evidence[13] for a similar reaction between nitroxides and semiquinones. We note that reactions of this kind are likely to lead to a less than quantitative yield of nitroxide in this and similar spin-trapping experiments. Radical generation in the presence of spin-labels similarly may lead to nitroxide loss.

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