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FORMATION AND REDUCTION OF A NITROXIDE RADICAL BY LIVER MICROSOMES

Gerald M. Rosen and Elmer J. Rauckman

Department of Physiology and Pharmacology, Duke University Medical Center,

Durham, N.C. 27710, U.S.A.

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Cytochrome P-450 is termed a mixed-function oxidase since it requires the presence of both a reducing agent and molecular oxygen (1). The ambivalent nature of this relatively non-specific enzyme system has led to its implication in reducing processes other than the reduction of molecular oxygen (2). The reductive intermediate, if cytochrome P-450 is to act as a reducing agent, is the ferrous form of the cytochrome in which to be reduced, the substrate replaces oxygen in its reaction with the ferrohemochrome.

Stable nitroxide radicals can be considered as oxygen analogs since they are paramagnetic, have relatively low reactivity under physiological conditions and have a terminal oxygen. Stier and Reitz (3) have reported that 2,2,6,6-tetramethylpiperidinoxyl (I) (Fig. 1) is reduced in the presence of liver microsomes, oxygen and NADPH. They also reported (4) that (I) could be formed enzymatically by the NADPH-dependent oxidation of 2,2,6,6-tetramethylpiperidine (II) in the presence of the microsomal enzymes and oxygen, and that a stable equilibrium concentration of (I) could be observed by electron paramagnetic resonance (e.p.r.) spectroscopy, indicating that an equilibrium between the oxidation of (II) and the reduction of (I) is achieved. Since Stier and Reitz (3,4) gave no experimental details, we decided to reinvestigate these reaction sequences.

The synthesis of 2,2,6,6-tetramethylpiperidinoxyl (I) was carried out according to the method of Rauckman *et al.* (5) by oxidation of 2,2,6,6-tetramethylpiperidine (II) using *m*-chloroperbenzoic acid. Reduction of (I) with platinum oxide and hydrogen gave 1-hydroxy-2,2,6,6-tetramethylpiperidine (III) (6).

Liver microsomes were prepared as reported in the literature (7). The microsomes were resuspended in 1.15% potassium chloride solution such that a final concentration of 0.5 g of whole liver/ml of suspension was obtained. The microsomal protein and cytochrome P-450 contents were determined by the methods of Lowry *et al.* (8) and Omura and Sato (9) respectively. For the drug induction studies, rats were injected intraperitoneally with phenobarbital (80 mg/kg) each day for 4 days. The liver microsomes were isolated as reported earlier.

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Electron paramagnetic resonance spectra were obtained using a Varian Associates model E-9 spectrometer. The kinetic studies were conducted by measuring the decrease or increase in the height of the central peak of the nitroxide triplet as a function of time. In a typical experiment, the reaction medium contained 5×10^{-5} M of the nitroxide or hydroxylamine (III), 2.5×10^{-4} M of NADPH, 0.1 ml of the microsomal enzymes and sufficient buffer (the solution contained 2.33×10^{-2} M KH_2PO_4 and 8.33×10^{-3} M $MgCl_2$ adjusted to pH 7.4) to bring the final volume to 0.5 ml. These experiments were conducted at ambient temperature in a 0.1 mm flat cell.

2,2,6,6-Tetramethylpiperidine (1×10^{-4} M) was incubated with the microsomal enzymes at ambient temperature in the presence of NADPH (2.5×10^{-4} M) and oxygen. Two controls were used: the first containing everything except NADPH, and the second containing everything except the microsomal enzymes. These mixtures were then placed in an e.p.r. spectrometer and the concentration of the nitroxide was determined.

Incubating 2,2,6,6-tetramethylpiperidine (II) in the presence of the microsomal enzymes, oxygen and NADPH did not increase the concentration of the nitroxide (I) over that of the control. This experiment was performed both by continuous observation of an oxygen-saturated solution in the e.p.r. cell for 30 min and by taking aliquots from a mixture through which oxygen was bubbled during a period of 1 hr. These experiments suggested that either the piperidine (II) is not oxidized to the nitroxide (I) via (III) or that the rate of reduction of (I) is greater than the rate of oxidation of (II) to (I). On the other hand, it is known that (III) undergoes relatively rapid autoxidation in the presence of air (10), suggesting that enzymatic oxidation of the amine (II) could be the rate-limiting step in the conversion of the amine (II) to the nitroxide (I).

Although we did not observe nitroxide formation from the amine (II), incubation of (III) with the microsomal enzymes and NADPH under aerobic conditions led to the formation of the nitroxide as evidenced by the appearance of its characteristic e.p.r. signal. This reaction was found to have a K_m of 3.2×10^{-4} M and V_{max} of 8.4×10^{-3} m-moles min^{-1} $\mu mole^{-1}$ of cytochrome P-450 (Fig. 2). We feel that the enzyme responsible for this oxidation is the mixed-function amine oxidase described by Ziegler and Mitchell (11). Evidence was gathered to demonstrate that cytochrome P-450 is not responsible for this oxidation: (1) the rate of oxidation is not increased using phenobarbital-induced microsomes, (2) the oxidation is not inhibited by SKF-525A as well as other specific inhibitors of cytochrome P-450, and (3) carbon monoxide only inhibited this reaction by 50 per cent when added to dithionite-reduced microsomes.

We have also observed that the nitroxide undergoes an NADPH-dependent reduction to the hydroxylamine (III) at a kinetically measurable rate with a K_m of 1.8×10^{-4} M and a V_{max} of 8.9×10^{-3} m-moles min^{-1} $\mu mole^{-1}$ of cytochrome P-450 (Fig. 3). This reduction is inhibited competitively by specific antagonists of cytochrome P-450. Pretreating the microsomal enzymes with a minimal amount of dithionite and then exposing the enzymes to carbon monoxide prior

to introducing the nitroxide and NADPH prevented the reduction of the free radical. On the other hand, this reduction was greatly increased if phenobarbital-induced microsomal enzymes were used in place of control enzymes.

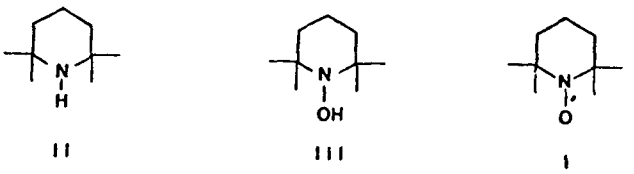

Fig. 1. Structure of the compounds investigated.

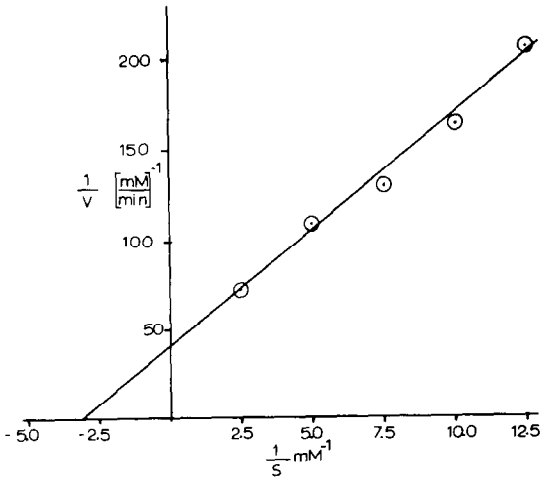

Fig. 2. Lineweaver-Burk plot for the oxidation of the hydroxylamine (III) to the nitroxide (I); K_m is 3.2×10^{-4} M; V_{max} is 8.4×10^{-3} m-moles/min/μmole.

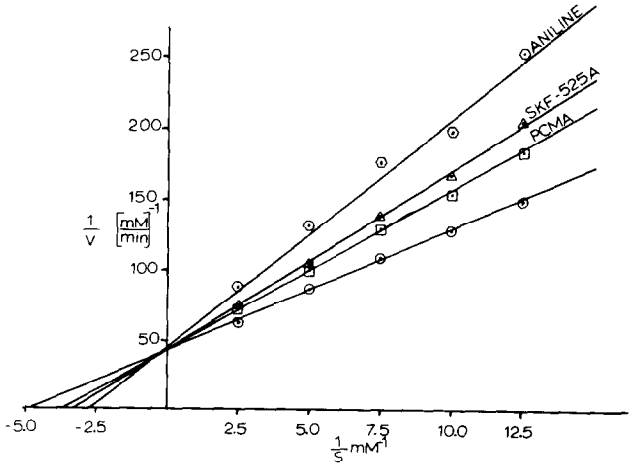

Fig. 3. Lineweaver-Burk plot for the inhibition of microsomal nitroxide reduction by various inhibitors: SKF-525A (0.018 mM) K_i is 3.3×10^{-5} M; PCMA (0.25 mM) K_i is 8.1×10^{-4} M; aniline (12.5 mM) K_i is 1.9×10^{-2} M. PCMA is p-chloro-N-methylaniline.

An alternative mechanism which would account for the loss of the nitroxide signal is alkyl oxidation of the piperidine ring and subsequent rearrangement to a non-radical product. To discount this possiblity, the product of the reduction was isolated by thin-layer chromatography and identified as the hydroxylamine (III).

In conclusion, we have shown that the hydroxylamine (III) is oxidized by the microsomal enzymes to the nitroxide (I) via an enzyme different than cytochrome P-450 and that cytochrome P-450 will reduce the nitroxide (I) to the hydroxylamine (III). These observations are consistent with the findings of Jenner et al.(12) and Das and Ziegler (13) who suggest that basic amines are N-oxidized by a non-cytochrome P-450-dependent system.

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