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Mechanism of Oxidation of *N*-Hydroxyphentermine by Superoxide†

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ABSTRACT: Cytochrome P-450 oxidizes *N*-hydroxyphentermine (MPPNHOH) by an indirect pathway involving superoxide. The chemical details of this oxidation, in which *N*-hydroxyphentermine is converted to 2-methyl-2-nitro-1-phenylpropane ($MPPNO_2$), have been elucidated by examining the interaction of MPPNHOH with superoxide in aqueous and organic solvents. The role of peroxide, hydroperoxy radicals, and oxygen in the reaction was also examined. The results indicate that superoxide itself is oxidizing MPPNHOH to a nitroxide that disproportionates to MPPNHOH and 2-methyl-2-nitroso-1-phenylpropane (MPPNO). MPPNO is then oxidized to $MPPNO_2$ by O_2 or hydroperoxide. Two possible mechanisms for the superoxide oxidation were considered, a proton abstraction and a hydrogen atom abstraction. Stoichiometric and oxygen evolution studies favor the hydrogen abstraction pathway.

Superoxide radical anion, $O_2^{-}\cdot$, is of interest in oxidations catalyzed by cytochrome P-450 because it is a potential by-product of some reactions. As it is both a radical and an anion, it has the potential for dual reactivity. As an anion, it can react as either a base or a nucleophile. Its reactivity is frequently dominated by its ability to act as a base. The conjugate acid of superoxide, HOO·, disproportionates via a bimolecular process to yield hydrogen peroxide and molecular oxygen, and the thermodynamics of this disproportionation has been proposed (Fee & Valentine, 1977) to be the basis for the effective pK_a of about 24 although the actual value is 4.69 in water (Sawyer & Valentine, 1981). In aprotic solvents, many of the oxidations attributed to superoxide can be accounted for by initial proton abstraction from the substrate to yield a substrate anion that is then oxidized by either O_2 or H_2O_2, the products of proton-induced disproportionation of superoxide (Nanni et al., 1980). The nucleophilic reactions of superoxide are usually seen only in aprotic, non-hydrogen-bonding solvents.

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Superoxide can also act as a radical, and it was believed that the radical character of superoxide was relatively unimportant in its reactions. However, recent studies of reactions of $O_2^{-}\cdot$ with substrates containing easily abstractable hydrogen atoms have demonstrated that superoxide can indeed behave as a hydrogen atom abstractor and effect one-electron oxidations of very weak bonds (Nanni & Sawyer, 1980; Rauckman et al., 1979).

Phentermine, 2-methyl-1-phenyl-2-propylamine ($MPPNH_2$)[1] is N-hydroxylated by a cytochrome P-450 dependent mechanism in rat and rabbit liver microsomes (Sum & Cho, 1977) and by a reconstituted cytochrome P-450 system (Duncan & Cho, 1982). *N*-Hydroxyphentermine (MPPNHOH) is then further oxidized by the enzyme system to yield 2-methyl-2-nitro-1-phenylpropane ($MPPNO_2$) (Scheme I) (Maynard & Cho, 1981), but unlike the N-hydroxylation the second oxidation was inhibited by superoxide dismutase (Sum & Cho, 1977, Duncan et al., 1985), suggesting participation by superoxide. In further support of this notion, the xanthine/xanthine oxidase system, which is known to produce superoxide, also was found to cause the oxidation of MPPNHOH (Sum & Cho, 1977; Duncan et al., 1985).

A proposed pathway for these observations is that $MPPNH_2$ is oxidized by cytochrome P-450 to MPPNHOH which binds to cytochrome P-450 and uncouples the cytochrome P-450 cycle to generate superoxide which chemically oxidizes MPPNHOH. The following study was undertaken to determine the mechanism of this superoxide oxidation of MPPNHOH. Exogenous superoxide in the form of either potassium superoxide (KO_2) or tetramethylammonium superoxide (NMe_4O_2) was used and its reaction with MPPNHOH studied in both aqueous and organic solvents. The results of this study and its relevance to the cytochrome P-450 mediated oxidation are the subjects of this paper.

Materials and Methods

All chemicals were purchased from commercial suppliers and used without further purification unless otherwise specified. Diethyl ether was purified by distillation from sodium benzophenone. All solutions were degassed by bubbling with nitrogen for at least 20 min. $MPPNH_2$ was obtained as a gift from Pennwalt Corp. (Rochester, NY). MPPNHOH was synthesized from $MPPNH_2$ according to Beckett et al. (1975), and 2-methyl-2-nitroso-1-phenylpropane (MPPNO) and $MPPNO_2$ were synthesized according to the method of Lindeke et al. (1975). Catalase was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN) and determined to be free of superoxide dismutase activity. Superoxide solutions were prepared either by saturation of dimethyl sulfoxide (Me_2SO) with KO_2 ($\sim$10 mM), solubilization of KO_2 to the desired concentration with 18-crown-6 (Valentine et al., 1984), or dissolution of NMe_4O_2. KO_2 (Alpha, Danvers, MA) was finely ground prior to use, and NMe_4O_2 was prepared by the method of Sawyer et al. (1983). Me_2SO was distilled from CaH_2 under vacuum. All reactions containing superoxide were performed in a glove bag with pure nitrogen atmosphere.

Product Analysis. The analysis of reaction products was conducted on an HPLC system consisting of a Milton-Roy minipump, Altex octadecylsilyl reverse-phase analytical column, and LKB 2138 Uvicord S UV–vis detector operating at 206 nm. The eluant was, in all cases, a 75:25 mixture of methanol–potassium phosphate buffer (50 mM, pH 2.8). When necessary for quantitation, 1-phenyl-2-hexanone was used as an internal standard, and response factors for MPPNO, $MPPNO_2$, and MPPNHOH relative to internal standard were determined (Duncan et al., 1985).

General Method for Reactions between Superoxide and MPPNHOH in Aqueous Solution. Under nitrogen, a solution of 487 000 units of catalase in 1.5 mL was added to a solution of MPPNHOH·HCl in 8.5 mL of degassed 0.5 M, pH 7.4, potassium phosphate buffer. The appropriate amount of the superoxide/Me_2SO solution was then slowly infused, with stirring, into the reaction mixture by using an all-glass syringe and a syringe pump. The reaction was quenched by the addition of 10 mL of a cold, degassed solution of 0.5 mM 1-phenyl-2-hexanone (internal standard) in methylene chloride. The biphasic solution was stirred vigorously in the glove bag for 20 min and centrifuged (2600 rpm for 10 min) and the aqueous layer discarded. The organic layer was evaporated by a stream of nitrogen to $\sim$0.5 mL. To the residue was added $\sim$1 mL of methanol and the mixture evaporated to 0.5 mL again. This was repeated once more or until most of the methylene chloride was gone. The methanolic solution was then analyzed by HPLC. Control experiments were run in parallel without superoxide.

Reaction between NMe_4O_2 and MPPNHOH at Varying pH Values. Under nitrogen, solutions of 487 000 units of catalase in 1.5 mL were added to solutions of 8.5 mL of 2.7 mM MPPNHOH in 0.5 M potassium phosphate buffer at pH values of 6.5, 7.0, 7.5, 8.5, and 9.0. To each solution was slowly infused, via an all-glass syringe, 0.5 mL of 14.3 mM NMe_4O_2 in Me_2SO. Workup as previously described yielded MPPNO in the following amounts: pH 6.5, 2.7% MPPNO; pH 7.0, 4.5% MPPNO; pH 7.5, 8.2% MPPNO; pH 8.5, 15.8% MPPNO; pH 9.0, 39.8% MPPNO. $MPPNO_2$ was formed in negligible amounts.

General Method for the Reaction between Superoxide and MPPNHOH in Ether. The appropriate amount of MPPNHOH·HCl was dissolved in a minimum amount of 0.01 M HCl ($\sim$1 mL). The solution was made basic with 0.2 N NaOH and extracted with 10 mL of ether. The ether solution was dried with $MgSO_4$ and filtered, and ether was removed by rotary evaporation. The residue was dried under vacuum, and the MPPNHOH was taken up in 10 mL of dry, degassed ether. In a glove bag, the appropriate amount of Me_2SO solution of either KO_2 or NMe_4O_2 was added to the ethereal MPPNHOH solution through an all-glass syringe. After stirring for 0.5 min, 5 mL of a solution of 1-phenyl-2-hexanone in methylene chloride was added, and the solution was washed with 3 × 10 mL of water. The organic phase was dried with $MgSO_4$, filtered, and evaporated to $\sim$0.5 mL with a stream of nitrogen. To the residue was added 1 mL of methanol, and the mixture was again evaporated to $\sim$0.5 mL. This step was repeated until most of the methylene chloride was removed. The remaining methanolic solution was analyzed by HPLC.

Oxidation of MPPNHOH with Hydrogen Peroxide with and without Added Fe^{II}. All the following manipulations were carried out in a glove bag with a nitrogen atmosphere. To a solution of 5 mg (2.5×10^{-5} mol) of MPPNHOH·HCl in 0.5 M, pH 7.4, phosphate buffer (10 mL) was added 100 μL of a 30% H_2O_2 solution. To another identical sample was added 100 μL of a 30% H_2O_2 solution and 7 mg (2.5×10^{-5} mol) of $Fe^{II}(SO_4)\cdot 7H_2O$. A third solution of 5 mg of MPPNHOH·HCl in buffer was used as a control. All three solutions were stirred for 0.5 h, and the solutions were analyzed

[1] Abbreviations: MPPNHOH, *N*-hydroxyphentermine; $MPPNO_2$, 2-methyl-2-nitro-1-phenylpropane; MPPNO, 2-methyl-2-nitroso-1-phenylpropane; $MPPNH_2$, 2-methyl-1-phenyl-2-propylamine; NMe_4O_2, tetramethylammonium superoxide; KO_2, potassium superoxide; Me_2SO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance.

Table I: Oxidation of MPPNHOH and MPPNO

substrate	solvent	$O_2^{-\cdot}$	H_2O_2	$H_2O_2(Fe^{II})$	HOO^-	TEMPO[a]	O_2	^-OH	base/O_2[b]
MPPNHOH[c]	aqueous	yes	yes	no[e]	yes	yes	slowly	no	no
MPPNHOH	ether	yes				yes	slowly		no
MPPNO[d]	methanol		no	no	yes		slowly	no	
MPPNO	ether	no	no				yes		

[a] TEMPO = 2,2,6,6-tetramethylpiperidinyloxy. [b] Bases used are KH, $NaNH_2$, butyllithium, and benzyltrimethylammonium hydroxide. [c] Oxidation to MPPNO. [d] Oxidation to $MPPNO_2$. [e] Some oxidation does occur, but it is less than with H_2O_2 itself.

directly by HPLC. The control reaction gave less than 4% conversion to MPPNO, the MPPNHOH·HCl–H_2O_2 mixture yielded 31% conversion to MPPNO, and the reaction performed in the presence of Fe^{II} gave 16.5% conversion to MPPNO.

Oxidation of MPPNHOH by Basic Hydrogen Peroxide. To a solution of 5 mg (2.5×10^{-3} mol) of MPPNHOH in water was added 1 mL of a 15% H_2O_2 in 0.1 N NaOH solution. This mixture and a control solution, to which only 1 mL of 0.1 N NaOH had been added, were stirred in a glove bag for 0.5 h. Analysis of the reaction mixture by HPLC revealed that ~47% oxidation to MPPNO had occurred in the basic H_2O_2/MPPNHOH solution. The control mixture had less than 3% oxidation to MPPNO.

Oxidation of MPPNO by Basic Hydrogen Peroxide. A solution of 20 mg of MPPNO in 40 mL of degassed methanol was divided into four equal portions. To one portion was added 1 mL of a 30% H_2O_2 solution, to another was added 1 mL of a 15% H_2O_2 in 0.1 N NaOH solution, to a third portion was added 1 mL of 0.1 N NaOH, and the final portion was left as a control. All four solutions were stirred under N_2 for 0.5 h. Analysis by HPLC of the solutions showed 54% oxidation to $MPPNO_2$ in the solution with added basic H_2O_2. All other solutions showed less than 5% oxidation to $MPPNO_2$.

Oxidation of MPPNHOH by Dibenzoyl Peroxide. To a 10-mL solution of degassed, pH 7.4, potassium phosphate buffer containing 5 mg (2.5×10^{-5} mol) of MPPNHOH·HCl was added 6 mg of dibenzoyl peroxide (2.5×10^{-5} mol). The resulting solution was heated in a glove bag for 15 min at 45 °C, then cooled to room temperature, and extracted with 10 mL of degassed methylene chloride. The organic extract was evaporated under a stream of nitrogen to 0.5 mL, then diluted with 1 mL of methanol, and again evaporated to ~0.5 mL. The methanolic solution was analyzed by HPLC. This procedure resulted in a 71% conversion of MPPNHOH to MPPNO.

Oxidation of MPPNHOH by 2,2,6,6-Tetramethylpiperidinyloxy (TEMPO). To solutions of 16.3 mg (9.9×10^{-5} mol) of MPPNHOH in 10 mL of dry, degassed ether was added varying equivalents of TEMPO. The resulting solutions were stirred under nitrogen for 2 h and then evaporated under a stream of nitrogen, and the residue was taken up in methanol. One equivalent of TEMPO resulted in a 49:51 ratio of MPPNHOH to MPPNO. One-half equivalent yielded a ratio of 69:31 and 2 equiv, a ratio of 4:96. The reaction was also performed with 1 equiv of TEMPO in pH 7.4 buffer as solvent, and the usual workup and HPLC analysis yielded a MPPNHOH:MPPNO ratio of 50:50.

Attempted Base/O_2 Oxidation of MPPNHOH. To a dry, degassed ethereal solution of 5 mM MPPNHOH was added, under N_2, 1 equiv of either potassium hydride, sodium amide, butyllithium, or benzyltrimethylammonium hydroxide. The resulting solution was then stirred for 5–10 min and then bubbled with pure O_2 gas for 15 min. After evaporation and resuspension in methanol, the mixture was analyzed by HPLC. No oxidized products, MPPNO or $MPPNO_2$, were ever observed.

Oxygen Detection during the Oxidation of MPPNHOH by Superoxide. All of the following manipulations were performed in a glove bag with a nitrogen atmosphere. Oxygen concentrations were measured with a YSI, Model 53, biological oxygen monitor equipped with an oxygen-permeable membrane. Dry ether saturated with O_2 by bubbling O_2 through the solvent for 0.5 h gave a large response by the electrode which represented 100% saturation (0.415 cm^3/mL O_2 in ether) (Fischer & Pfleiderer, 1922). Dry, degassed ether and wet, degassed ether (saturated aqueous ether, ~1.26% H_2O) showed no electrode responses. When 0.50 mL of a 0.1 M solution of NMe_4O_2 in Me_2SO was added to dry ether, no response was registered by the electrode. However, when 0.5 mL of the 0.1 M NMe_4O_2 in Me_2SO solution was added to wet ether, a large response was observed that corresponded to 34% saturation. The theoretical response is 38%, based on O_2 concentration in O_2-saturated ether. When a 0.50-mL solution of 0.1 M tetramethylammonium superoxide in Me_2SO (5×10^{-5} mol) was added to 8 mg (5×10^{-5} mol) of MPPNHOH in 5 mL of dry, degassed ether, no response by the electrode was observed. Standard workup and HPLC analysis of the sample showed that ~73% of the added substrate was accountable as the oxidized products, MPPNO and $MPPNO_2$.

Results

Initial experiments were designed to determine at what stage(s) in the overall oxidation superoxide participates and what other, if any, oxidants are present and active. Enzymatic studies indicated that the oxidation of $MPPNH_2$ to MPPNHOH is not superoxide mediated. The oxidation of MPPNHOH to $MPPNO_2$ is not necessarily a one-step process and could involve a nitroso, MPPNO, or other intermediate, but the assay system used in the initial studies (Sum & Cho, 1977) measured only $MPPNO_2$. Therefore, an HPLC assay was developed that permitted the analysis of MPPNHOH, MPPNO, and $MPPNO_2$ simultaneously (Duncan et al., 1985). By use of this assay, it was found that reaction between MPPNHOH and KO_2 in anaerobic conditions in either aqueous buffer or diethyl ether yields MPPNO (Table I) and small amounts of $MPPNO_2$. MPPNO is usually the primary product and, when exposed to air, slowly autoxidizes to $MPPNO_2$. These results are paralleled in the enzymatic systems where both MPPNO and $MPPNO_2$ are detectable as metabolites in either reconstituted cytochrome P-450 or xanthine/xanthine oxidase systems. The formation of both compounds is inhibitable by superoxide dismutase (Duncan et al., 1985). A summary of the oxidations attempted is presented in Table I.

The autoxidation of MPPNO to $MPPNO_2$ is more rapid in organic solvents than in water presumably due to either differing oxygen solubility in aqueous vs. organic solvent or, as is more likely, a displacement of the monomer–dimer equilibrium. Nitroso compounds are known to form dimers in the solid phase and exist as both monomer and dimer in solution (Coombes, 1979). Preliminary experiments indicate that only the monomer is capable of being oxidized so that

the equilibrium concentration of monomeric MPPNO controls the rate of autoxidation.

These initial studies showed that MPPNO was an intermediate in both the chemical and biochemical systems and that superoxide was involved in its production from MPPNHOH. The subsequent oxidation of MPPNO was *not* caused by a direct reaction with $O_2^{-}\cdot$ since no oxidation occurred when MPPNO was mixed with KO_2 (Me_2SO solution) in diethyl ether. Some oxidation does occur during workup, but both the reaction mixtures and the control mixtures, which lack $O_2^{-}\cdot$, have identical amounts of $MPPNO_2$.

Since superoxide can disproportionate to hydrogen peroxide and molecular oxygen, it is conceivable that these products or others generated from them can participate in or contribute to the overall oxidative pathway. For this reason, other possible oxidants were examined for their ability to participate in the reactions.

Hydrogen peroxide will slowly oxidize MPPNHOH to MPPNO but will not effect the subsequent oxidation of MPPNO to $MPPNO_2$ (Table I). If 0.100 mL of 30% aqueous H_2O_2 is added, under nitrogen, to a 10-mL solution of 5 mM MPPNHOH in degassed, pH 7.4, potassium phosphate buffer, as much as 31% oxidation to MPPNO occurs after 0.5 h. This conversion is not enhanced with the addition of Fe^{II} and EDTA. However, the presence of large amounts of catalase in the actual superoxide-mediated oxidations precludes this as a major oxidative pathway. MPPNO will not oxidize under any of these conditions (Table I).

Molecular oxygen will slowly oxidize both MPPNHOH and MPPNO, but the rate of oxidation of both forms appears to be so much slower than with other oxidants that it cannot be considered as a primary oxidant. In enzymatic systems, however, it could be a more important pathway.

The above experiments indicated that superoxide participation in the overall oxidative process is primarily in the oxidation of MPPNHOH to MPPNO (Scheme I). Molecular oxygen does not appear to be important in the conversion of MPPNHOH to MPPNO. The next series of experiments were conducted to determine the mechanistic details of the superoxide-mediated oxidation of MPPNHOH. The action of superoxide in aqueous and organic solvents was assessed to determine the mechanism by which superoxide reacts with MPPNHOH and the oxidant(s) responsible for further oxidation.

Scheme I

$$MPPNH_2 \xrightarrow{P\text{-}450} MPPNHOH \xrightarrow{O_2^{-}\cdot} MPPNO \xrightarrow{[o]} MPPNO_2$$

MPP = $C_6H_5CH_2C(CH_3)_2$–; [o] = O_2 or HOO^-

The reaction between MPPNHOH and KO_2 was investigated in pH 7.4 buffer solution in the presence of catalase (487 500 units). The amount of catalase used exceeded that required to inhibit any oxidation of MPPNHOH by hydrogen peroxide which would result from the complete disproportionation of added KO_2.[2] Very little oxidation of MPPNHOH occurred when a 10 mM solution of KO_2 (Me_2SO solution) was slowly infused into a 0.1 mM solution of MPPNHOH in buffer. A likely explanation for the poor conversion was loss of superoxide by disproportionation, a rapid proton-induced bimolecular process that generates both hydrogen peroxide and oxygen (Fee & Valentine, 1977). An attempt was then made to "trap" superoxide with high substrate concentrations before it could disproportionate. Thus, 0.5 mL of a 10 mM solution of KO_2 in Me_2SO was slowly infused into a series of buffered solutions with increasing concentrations of MPPNHOH and added catalase. Increasing substrate concentrations increased the yield, based on KO_2, of oxidized product with a maximal conversion of 25%. Higher conversions were impossible due to the insolubility of MPPNHOH at concentrations greater than ~5 mM. Thus, high substrate concentrations can trap some superoxide before it undergoes disproportionation, and under these conditions superoxide-mediated oxidation of MPPNHOH was shown to occur even in aqueous systems.

The reaction between NMe_4O_2 and MPPNHOH was also performed at varying pH. Thus, 0.5 mL of 14.3 mM $O_2^{-}\cdot$ was infused into solutions of 2.7 mM MPPNHOH, with added catalase, at pH 6.5, 7.0, 7.5, 8.5, and 9.0. As the pH of the solution is increased, the yield of MPPNO is increased: pH 6.5, 2.7%; pH 7.0, 4.5%; pH 7.5, 8.2%; pH 8.5, 15.8%; pH 9.0, 39.4%. This phenomenon can be attributed to the increased lifetime of superoxide at higher pH. It should be noted that catalase is equally active at all the pH values used (Chance, 1952), thus precluding hydrogen peroxide participation. Also, the pK_a of the conjugate acid of MPPNHOH is 5.5 (Jonsson et al., 1977) so that all solutions contained primarily the free base form.

The oxidation of MPPNHOH by KO_2 in an organic solvent was also investigated. When 2 equiv of a 10 mM solution of KO_2 in Me_2SO was added, under nitrogen, to a dry, degassed ether solution of MPPNHOH, greater than 94% total oxidized products were obtained after 0.25 h (63% MPPNO and 32% $MPPNO_2$). The greater percentage of oxidation in ether compared to aqueous solutions is most likely due to the absence of solvent-induced disproportionation of superoxide. The results of these studies show that superoxide-mediated oxidation of MPPNHOH is possible in both organic and aqueous solutions. However, the mechanism of the oxidations may not necessarily be the same.

One possible mechanism reflects the ability of superoxide to act as a base (Sawyer & Valentine, 1981) and involves initial proton abstraction by superoxide and subsequent oxidation of the substrate anion by either O_2 or H_2O_2 (Scheme II). In a second possibility, superoxide acting as a *radical* may abstract a hydrogen atom from MPPNHOH to generate a nitroxide intermediate that disproportionates to MPPNO which is then further oxidized in solution to $MPPNO_2$ (Scheme III). In pH 7.4 aqueous buffer systems, the reaction path involving proton abstraction (reaction 1 in Scheme II) cannot occur since, with a pK_a of 4.69 for HOO·, superoxide is not a strong enough base. In diethyl ether, however, either mechanism can occur since $O_2^{-}\cdot$ would be expected to be a very strong base in this solvent.

Scheme II

$$MPPNHOH + O_2^{-}\cdot \rightarrow MPPNHO^- + HOO\cdot \quad (1)$$

$$HOO\cdot \rightarrow {}^1/_2O_2 + {}^1/_2H_2O_2 \quad (2)$$

$$HOO\cdot + O_2^{-}\cdot \rightarrow HOO^- + O_2 \quad (3)$$

$$MPPNHO^- + H_2O_2 \rightarrow \text{oxidized products} \quad (4)$$

$$MPPNHOH + HOO^- \rightarrow \text{oxidized products} \quad (5)$$

[2] Kono & Fridovich (1983) have reported that superoxide can inactivate catalase to an apparent limit of 50%. However, the large excess of catalase used in these studies was such that any catalase inactivation due to the infusion of KO_2 into solutions would not significantly alter the results.

Scheme III

$$\text{MPPNHOH} + O_2^{-}\cdot \rightarrow \text{MPPNHO}\cdot + \text{HOO}^- \quad (6)$$

$$\text{MPPNHO}\cdot \rightarrow {}^1/_2\text{MPPNHOH} + {}^1/_2\text{MPPNO} \quad (7)$$

$$\text{MPPNO} + O_2 \rightarrow \text{MPPNO}_2 \quad (8)$$

$$\text{MPPNHOH} + \text{HOO}^- \rightarrow \text{MPPNO} + H_2O + {}^-\text{OH} \quad (9)$$

$$\text{MPPNO} + \text{HOO}^- \rightarrow \text{MPPNO}_2 + {}^-\text{OH} \quad (10)$$

The validity of the proposed radical pathway in aqueous systems was tested by using other nonbasic, potential hydrogen atom abstractors. Dibenzoyl peroxide and TEMPO oxidized MPPNHOH to MPPNO, and if 1 equiv of TEMPO was used, an equimolar mixture of MPPNO and MPPNHOH was obtained as the stoichiometry of reaction 7 predicts (Table II). The oxidation by these agents occurred in ether as well, and varying equivalents of TEMPO gave the approximate stoichiometry predicted for the radical mechanism (Table II). On the basis of these data, the radical reaction, shown as reaction 6 in Scheme III, is proposed in both aqueous and organic solvents with subsequent disproportionation of the nitroxide, according to reaction 7 in Scheme III, to MPPNO. This disproportionation reaction is not without literature precedent. In fact, the reverse of this type of reaction has been used to generate ESR-detectable concentrations of primary nitroxides from mixtures of the corresponding nitroso and *N*-hydroxy compounds (Wajer et al., 1969).

These data demonstrate that hydrogen atom abstraction can lead to the observed oxidized products, but they do not show that superoxide is reacting by this pathway. If superoxide did abstract a hydrogen atom from MPPNHOH, it would become a hydroperoxide anion, HOO^-. Hydrogen peroxide itself has already been shown to slowly oxidize MPPNHOH but not MPPNO. However, HOO^- will oxidize both MPPNHOH and MPPNO (Table I). In 30 min, aqueous HOO^- converted MPPNHOH in aqueous solution to a mixture of 40% MPPNO and 7% $MPPNO_2$ and converted MPPNO in methanol to 54% $MPPNO_2$. These data indicate that a superoxide-mediated pathway for the production of $MPPNO_2$ from MPPNHOH under anaerobic conditions is possible (reactions 6, 7, 9, and 10 in Scheme III).

The possibility that proton abstraction and oxygen oxidation of MPPNHOH may occur in ether was tested with nonradical bases. Potassium hydride, sodium amide, butyllithium, or benzyltrimethylammonium hydroxide were added to ethereal solutions of MPPNHOH, and upon formation of the substrate anion, MPP–NHO^-, oxygen was bubbled through the solution. No oxidation occurred in any instance so that oxidation of the anion by molecular oxygen was deemed not to be a major pathway. However, the reaction of the anion with hydrogen peroxide was still a possibility. This reaction is equivalent to the reaction of HOO^- with neutral MPPNHOH (reaction 9) and could not be unambiguously ruled out with these data.

One distinguishing characteristic between the two mechanisms is that O_2 is evolved in the base-mediated reaction (reactions 2 and 3 in Scheme II) but is not in the radical-mediated pathway (Scheme III). Therefore, oxygen evolution was determined with an oxygen electrode during the reaction of superoxide with MPPNHOH in ether. The results show that no oxygen was detected even though 75% total oxidized products were obtained from the reaction of one equivalent of KO_2 with MPPNHOH in ether. Therefore, the oxidation occurs by a mechanism that does *not* liberate oxygen.[3]

Table II: Oxidation of MPPNHOH with TEMPO[a]

solvent	equiv of TEMPO	% conversion[b] of MPPNO	
		found	predicted[c]
diethyl ether	0.5	31	25
diethyl ether	1.0	51	50
diethyl ether	2.0	96	100
water	1.0	50	50

[a] TEMPO = 2,2,6,6-tetramethylpiperidinyloxy. [b] The remainder of the material in solution is starting material, MPPNHOH. [c] Based on the stoichiometry of reaction 7 in Scheme III.

Table III: Stoichiometry[a] of the Superoxide Oxidation of MPPNHOH in Ether

equiv of $O_2^{-}\cdot$	% MPPN-HOH[b]	% MPPNO	% $MPPNO_2$	% total oxidized product[c]
0.5	48	31	21	52
1	25	34	41	75
2	22	3	75	78

[a] Stoichiometries are only approximate since optimal reaction conditions were not determined. [b] Mole percent starting material remaining at the end of the reaction. [c] Mole percent MPPNO plus $MPPNO_2$.

Another distinguishing characteristic between the two mechanisms is the stoichiometry of the overall oxidative process. The base-mediated mechanism would yield maximally 0.5 equiv of oxidized product, MPPNO, per equiv of superoxide since only 0.5 equiv of H_2O_2 is generated (reaction 2) and the other product, O_2, has already been shown not to perform the oxidation. In the radical-mediated mechanism, 1 equiv of superoxide should yield between 0.5 and 1.0 equiv of oxidized products depending on the relative rates of reactions 9 and 10 in Scheme III. One-half equivalent of superoxide would give between 0.5 and 0.75 equiv of total oxidized products. The results of the stoichiometry studies, as shown in Table III, indicate that the stoichiometry of the reaction of superoxide with MPPNHOH is more nearly consistent with the radical mechanism since product levels were too high for the base-mediated mechanism to be valid in all cases.

Discussion

As mentioned earlier, superoxide is frequently observed in organic solvents to react as a strong base so it is not unreasonable to propose oxidative mechanisms that involve initial proton abstraction (Scheme II). Furthermore, if the active site of cytochrome P-450 is indeed nonpolar and aprotic, su-

[3] A referee has proposed a base-mediated mechanism that will not liberate molecular oxygen:

$$O_2^{-}\cdot + \text{MPPNHOH} \rightarrow \text{MPPNHO}^- + \text{HOO}\cdot \quad (a)$$

$$\text{HOO}\cdot + \text{MPPNHOH} \rightarrow H_2O_2 + \text{MPPNHO}\cdot \text{ etc.} \quad (b)$$

This mechanism requires reaction b to be significantly faster than other O_2 producing reactions (Sawyer and Gibian, 1979):

$$\text{HOO}\cdot + \text{HOO}\cdot \rightarrow H_2O_2 + O_2 \qquad R = 7.6 \times 10^5\ \text{m}^{-1}\ \text{s}^{-1}$$

$$\text{HOO}\cdot + O_2^{-}\cdot \rightarrow \text{HOO}^- + O_2 \qquad R = 8.5 \times 10^7\ \text{m}^{-1}\ \text{s}^{-1}$$

However, the rates of reactions similar to (b), the hydrogen atom abstraction of secondary hydroxylamines by alkyl peroxides, are much slower than the reaction of $\text{HOO}\cdot + O_2^{-}\cdot$ (Denisov, 1974). Even when the increased reactivity of HOO· compared to alkyl peroxide and the solvent differences are considered, the rates of such reactions at these temperatures should be on the order of 10^4–$10^6\ \text{m}^{-1}\ \text{s}^{-1}$. The O_2 assay is capable of detecting about 1% O_2 evolution which would require the rate of reaction b to be $\sim 10^9$–$10^{10}\ \text{m}^{-1}\ \text{s}^{-1}$ which is unlikely. However, our results cannot unequivocally rule out this mechanism.

peroxide would be a strong base and could react accordingly. However, an oxidative mechanism involving proton abstraction by superoxide can be ruled out since (1) oxidation by other strong bases and oxygen does not occur, (2) the stoichiometry of such a process is inconsistent with that observed, and (3) no oxygen is evolved during the oxidative process. If the oxidation does occur by a base-mediated mechanism, appreciable amounts of O_2 should be generated.

The alternative mechanism in which superoxide acts as a radical and abstracts a hydrogen atom is the likely oxidative pathway, and consistent with this notion, other nonbasic radical sources such as dibenzoyl peroxide or TEMPO were able to perform the oxidation of MPPNHOH to MPPNO. This study also showed that other oxidants that would be present in the reaction mixtures containing superoxide such as the HOO^- can further react with MPPNO to eventually yield $MPPNO_2$.

Thus, we conclude that the oxidation of MPPNHOH by superoxide in either aqueous or organic media occurs via a hydrogen atom abstraction to generate a nitroxide (reaction 6) that disproportionates to give MPPNO and MPPNHOH (reaction 7). In the hydrogen atom abstraction reaction superoxide is converted to HOO^- which can effect further oxidation of either MPPNHOH or MPPNO (reactions 9 and 10).

Most previously reported oxidations by superoxide involve initial proton abstraction, generating a substrate anion which is subsequently oxidized by O_2 or H_2O_2 (Sawyer & Valentine, 1981). However, hydrogen atom abstraction by superoxide as proposed here does have literature precedence. Nanni & Sawyer (1980) have reported that the oxidation of hydroxylamine, hydroxyphenazines, reduced flavins, nicotinamides, and hydrazine by superoxide occurs via an initial hydrogen atom transfer from the substrate to superoxide. Also, on the basis of the calculated H–O bond energy for HOO^-, Liebman & Valentine (1984) have stated that though superoxide is relatively unreactive as a free radical, it can act as a hydrogen atom abstractor in the cases of compounds containing labile hydrogens. MPPNHOH, like hydroxylamine itself, is such a compound as are the reduced flavins, nicotinamides, etc. studied by Nanni & Sawyer (1980).

Hydrogen atom abstraction by superoxide has also been implicated in biochemical systems. Rosen & Rauckman (1977) showed by ESR techniques that 1-hydroxy-2,2,6,6-tetramethylpiperidine was converted to the nitroxide in incubation mixtures of liver microsomes. This oxidation was later found to be mediated by the flavoprotein mixed-function amine oxidase, and the oxidizing agent was shown to be superoxide (Rauckman et al., 1979). Thus, in both chemical and enzymatic systems, superoxide is capable of abstracting labile hydrogen atoms from hydroxylamines.

Although much of the data presented here are from experiments performed in diethyl ether, the conclusion may be relevant to cytochrome P-450 mediated oxidations since there is evidence that its active site is nonpolar. For example, cytochrome P-450 will only bind a nitrogen-containing substrate in its free base form (Cho & Miwa, 1974). Also, a positive correlation between lipophilicity and the K_m of the N-demethylation of substrates has also been noted (Duncan et al., 1983). On the basis of these observations, it is not unreasonable to view the active site of cytochrome P-450 as a nonpolar environment. In this environment, the superoxide generated might have an appreciable lifetime, since most water molecules would be excluded. In fact, an oxidative mechanism in a nonpolar environment could be fundamentally different from the pathway occurring in aqueous systems. However, it appears that the oxidation in both organic and aqueous solution occurs via the same mechanism.

Hydrogen peroxide is produced from the spontaneous disproportionation of superoxide, but it is not the primary oxidant of MPPNHOH by the cytochrome P-450 system. It may play a minor role in the observed oxidation since the addition of catalase to the incubation mixture of cytochrome P-450 will partially inhibit formation of oxidized products (Duncan et al., 1985). However, catalase will not inhibit the oxidation of MPPNHOH by the xanthine/xanthine oxidase system (Duncan et al., 1985), and the glucose/glucose oxidase system, which catalytically produces hydrogen peroxide, will not perform the oxidation of MPPNHOH (Maynard & Cho, 1979). Thus, although the exact role or contribution of hydrogen peroxide to the overall oxidative process is unclear in the enzymatic systems, it is apparent that superoxide itself is responsible for most of the oxidation of MPPNHOH.

It is worth noting that, in aqueous solution, the possibility exists that the oxidation of MPPNHOH is mediated by a trace metal which catalyzes an autoxidation process as has been demonstrated in hydroxylamine oxidations (Kono, 1978, Elstner & Heupel, 1976). This possibility, and potential problem, has been previously addressed by Rosen and coworkers (1982). They demonstrated that indeed trace metals can perform a one-electron oxidation of arylhydroxylamines generating the corresponding nitroxide, but this reaction can be sequestered with chelating agents. With artifactual oxidation by metals reduced to negligible amounts, they found that superoxide *will* abstract a hydrogen atom from alkylhydroxylamines, in an analogous reaction to that proposed here, to generate alkyl nitroxides. In our study, the nitroxides react further, whereas in their study, the nitroxides were stable and could be observed via ESR.

The mechanistic conclusions drawn here are meant to apply to the cytochrome P-450 mediated oxidation of MPPNHOH as well as the simple chemical system. The results presented here have been paralleled with studies performed with the actual biochemical system (Duncan et al., 1985). Both investigations show (1) that superoxide is directly involved in the oxidation of MPPNHOH, (2) that the reaction follows the sequences presented in Scheme I, and (3) that superoxide is involved specifically in the oxidation of MPPNHOH and MPPNO and not MPPNO to $MPPNO_2$. Since the mechanism of oxidation by superoxide is seemingly the same in both organic and aqueous environments, Scheme III represents the most probable mechanistic pathway for superoxide-mediated oxidation of MPPNHOH in chemical and enzymatic systems. The relative importance of reactions 8, 9, and 10 might differ substantially between the chemical system and the P-450 enzyme, due to differences in the availability of protons or oxygen and the intrinsic binding factors of the various compounds. Nevertheless, the general overall mechanism prescribed here appears entirely reasonable in both the biochemical and chemical systems.

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Registry No. MPPNHOH, 38473-30-2; MPPNO, 52497-67-3; $MPPNO_2$, 34405-43-1; TEMPO, 2564-83-2; superoxide, 11062-77-4; cytochrome P-450, 9035-51-2; monooxygenase, 9038-14-6.

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Multiple Copies of Phosphorylated Filaggrin in Epidermal Profilaggrin Demonstrated by Analysis of Tryptic Peptides†

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ABSTRACT: The precursor of mouse (c57/B16) epidermal filaggrin (profilaggrin) is a very large (ca. 500 000 daltons), highly phosphorylated protein containing multiple copies of filaggrin (26 000 daltons). The conversion of profilaggrin to filaggrin late in epidermal cell differentiation involves dephosphorylation and proteolysis to yield the unphosphorylated filaggrin, which polymerizes with keratin filaments into macrofibrils. In order to gain insight into the nature of these processes, we compared tryptic digests of profilaggrin with those of filaggrin by reverse-phase liquid chromatography. Approximately 80% of the profilaggrin mass consists of multiple copies of filaggrin. Twenty peptides purified in good yield from both profilaggrin and filaggrin accounted for most of the filaggrin sequence. A detailed analysis of the yield of several peptides provided an estimate of the size and frequency of the repeat unit within profilaggrin. These data indicate that the repeating substructure of profilaggrin contains about 265 amino acids and that about 50 residues are removed per filaggrin domain as the precursor is processed to filaggrin. Assuming a molecular weight of 500 000 (as estimated from sodium dodecyl sulfate–polyacrylamide gel electrophoresis), this indicates there are 16 repeats. Analysis of phosphopeptides isolated from profilaggrin showed that 66% of the phosphate was located on peptides that are unphosphorylated in filaggrin. Analysis of peptide recoveries confirmed the repeat size and showed that every copy of filaggrin was phosphorylated in profilaggrin.

Epidermis is a stratified tissue with dividing basal cells that differentiate sequentially into nucleated spinous and granular cells and finally into the anucleate cells of the stratum corneum [cf. review by Odland (1983)]. This keratinization process involves reorganization of the keratin filaments of the cells. A cationic histidine-rich protein called filaggrin (also called HRP or stratum corneum basic protein, SCBP) has been isolated from rat (Dale, 1977), mouse (Steinert et al., 1981), and human (Lynley & Dale, 1983) stratum corneum and appears to interact with keratin filaments at the time of terminal differentiation (cornification) to aid in the alignment of the keratin filaments in the cornified cells (Dale et al., 1978). Filaggrin aggregates keratin filaments in vitro, forming macrofibrils with a morphology similar to that seen in the stratum corneum (Dale et al., 1978) and a stoichiometry of two filaggrin molecules for each three keratin molecules (Steinert et al., 1981).

In neonatal mouse, filaggrin has a molecular weight of 25 840 (Steinert et al., 1981) and arises in vivo from a much larger (ca. 500 000 daltons), highly phosphorylated precursor

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