Reactive Species Produced by the 5-Methylphenazinium Methyl Sulfate/Reduced β-Nicotinamide Adenine Dinucleotide/Oxygen System in the Hydroxylation of Benzoic Acid

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Abstract: The hydroxylation of benzoic acid induced by the aqueous chemical system composed of 5-methylphenazinium methyl sulfate, reduced β-nicotinamide adenine dinucleotide (NADH), and dissolved oxygen has been investigated. A quantitative determination of the reaction products was made using high-pressure liquid chromatographic methods. The effect of reaction conditions and of additives on the product yields was examined. Results indicate that the species initiating hydroxylation is the hydroxyl radical, produced from a one-electron reduction of hydrogen peroxide in an adventitions-metal-ion catalyzed, Fenton-type reaction. Hydrogen peroxide is thought to be produced directly by the transfer of a hydride ion from a fully reduced 5-methylphenazinium cation to molecular oxygen, not by the disproportionation of superoxide radicals. It is apparent that superoxide radicals are produced to a very limited extent during reaction and do not contribute substantially to product formation. The sole function of NADH is the reduction of the 5-methylphenazinium cation.

Introduction

The aqueous chemical system composed of 5-methylphenazinium methyl sulfate (phenazine methosulfate), reduced β-nicotinamide adenine dinucleotide (NADH), and dissolved oxygen is known to hydroxylate a variety of aromatic compounds.[1-3] Generally, it has been assumed that superoxide radicals ($O_2^{-}\cdot$) are generated when the 5-methylphenazinium cation (MP^+), reduced by NADH, is reoxidized by oxygen.[1] Furthermore, it has been assumed that the reactive species initiating hydroxylation in the $MP^+/NADH/O_2$ system is $O_2^{-}\cdot$.[2] The $MP^+/NADH/O_2$ system is often cited as an example of a chemical system which produces $O_2^{-}\cdot$ (e.g., ref 4-7), and it has been used as a purported generator of $O_2^{-}\cdot$ in a frequently cited[8] investigation of the mechanism of action of the enzyme dopamine-β-hydroxylase (EC1.14.17.1).[9] In addition, MP^+ is commonly employed as an electron carrier in biological studies[10] where both oxygen and reducing agents are present.

In contrast to the findings of previous investigators, Halliwell[3] observed that the hydroxylation of *o*- or *p*-hydroxybenzoic acid by the $MP^+/NADH/O_2$ system was not affected by the addition of superoxide dismutase (EC1.15.1.1), and that catalase (EC1.11.1.6), which decomposes hydrogen peroxide to water and O_2, was an effective inhibitor. Thus, it appears that the hydroxylation reaction requires H_2O_2, but not $O_2^{-}\cdot$. In view of these findings, we have reexamined the hydroxylation of benzoic acid by the $MP^+/NADH/O_2$ system in order to identify products and clarify the reaction mechanism. Benzoic acid was chosen as the substrate since its hydroxylation by this system has been reported,[2] and the product yields of its reaction with hydroxyl radical ($HO\cdot$) have been carefully determined by Schuler and co-workers.[11] Here, we report results of investigations which have utilized high-pressure liquid chromatographic (LC) techniques to separate, identify, and quantify the products of the hydroxylation of benozic acid by the $MP^+/NADH/O_2$ system.

Experimental Section

Materials. 5-Methylphenazinium methyl sulfate (phenazine methosulfate; Aldrich Chemical and Sigma Chemical), β-nicotinamide adenine dinucleotide, reduced form (NADH; Sigma Stock No. N8129), *N,N*-bis[2-[bis(carboxymethyl)amino]ethyl]glycine (DETAPAC; "Baker" grade, ≥98%), sodium acetate (Fluka AG, Puriss p.a.), and tetrabutylammonium perchlorate (Alfa Products, Baker, and Eastman Kodak) were used as received. *m*-Toluic acid (Aldrich) was recrystallized from triply distilled water until LC detectable impurities were eliminated. Water was distilled serially from acid dichromate, alkaline permanganate, and pure water. All other chemicals were reagent grade from Baker Chemical or Fisher Scientific.

Experiments. All reactions were carried out under dim red lighting at room temperature. Hydroxylations were initiated by adding NADH, the limiting reagent, to solutions containing (excess) benzoic acid, 5-methylphenazinium (MP^+), buffer, additives, and O_2. The concentrations of the NADH stock solutions (prepared immediately before use) were determined by measurement of the 338-nm absorbance of pH 10 buffer solutions.[12] Reaction solutions were either exposed to air and continually stirred, or placed in capped vessels and mixed only after additions of MP^+ and NADH. For variation of $[O_2]$, aliquots of O_2- and N_2-saturated solutions were combined. The completion of reaction was determined visually from color changes indicative of the oxidation state of the MP^+.[13] Immediately after addition of NADH, the yellow solutions became chartreuse or green. Gradually, they returned to the original yellow color. The reaction was quenched after a maximum conversion of the benzoic acid of 4% by adding HCl (and *m*-toluic acid as an internal standard). Unreacted benzoic acid and reaction products were extracted with ether and identified by comparison of high-pressure LC retention times with commercial samples. Assignments were verified by IR spectroscopic analysis.

Chromatography. Analyses were performed using a high-pressure LC system consisting of a Perkin-Elmer Series 1 LC pump, Rheodyne loop injector, Waters Model 440 absorbance detector, Leeds & Northrup

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Table I. Dependence of Product Yields on pH[a]

pH	$[HBA]_f/[NADH]_0$	relative yields oHBA	mHBA	pHBA
4.3	0.206 ± 0.002	1.00	0.785	0.474
4.5	0.172 ± 0.020	1.00	0.753	0.511
4.7	0.138 ± 0.006	1.00	0.788	0.504
		av 1.00	0.775 ± 0.009	0.496 ± 0.009

[a] Initial reactant concentrations: citrate buffer, 50 mM; BA, 5mM; MP^+, 250 μM; NADH, 310 μM. Initially air-saturated solutions were open to the air and stirred. Data are averages of two identical sets of experiments.

Speedomax Type G recorder with disk integrator, and Hewlett-Packard Model 3390-A reporting integrator. Reversed-phase ion-pair chromatography was employed by using a Waters μ-Bondapak C_{18} analytical column (0.39 × 30 cm) and a mobile phase of water/methanol, 50 mM phosphate buffer pH 7.5, and 10 mM tetrabutylammonium perchlorate. Peaks were monitored with 254- or 280-nm UV absorbance and their areas determined by disk or electronic integration.

Spectroscopy. IR spectra were recorded on a Perkin-Elmer CDS/2 which consists of a Model 580 IR spectrophotometer, Interdata 6/16 minicomputer, and Interdata teletypewriter. IR spectra of commercial *m*- and *p*-hydroxybenzoic acid were obtained from a single scan of a solvent-cast film on a NaCl crystal. Spectra of commerical *o*-hydroxybenzoic acid and the collected reaction products (ca. 1 mg) were obtained from solvent-cast films on a ThBr (KRS-5) crystal using a multiple internal reflectance (MIR) accessory.

UV-absorption spectra were obtained on a Perkin-Elmer Model 575 absorbance spectrophotometer.

Results

In the hydroxylation of aromatic substrates by the 5-methylphenazinium methyl sulfate/reduced β-nicotinamide adenine dinucleotide(NADH)/O_2 system, NADH serves as an electron source and the 5-methylphenazinium cation (MP^+) as an electron carrier. Presumably, O_2 is split with one atom being incorporated into the substrate and the other being reduced to H_2O. In the present experiments, the hydroxylation of benzoic acid (BA) by this system is examined. In each case, benzoic acid was in substantial excess with respect to the NADH so that the limiting reagent was, in most cases, the NADH. The maximum conversion of benzoic acid was 4%, so that reactions with products are negligible.

High-pressure LC analysis indicates that *o*-, *m*-, and *p*-hydroxybenzoic acid (oHBA, mHBA, and pHBA, respectively) are formed as major products in the hydroxylation of benzoic acid by the $MP^+/NADH/O_2$ system. Identities of the products, as indicated by LC retention times, were confirmed by IR spectroscopic analyses of the eluted peaks. For each isomer, at least 14 bands in the 650–1800-cm^{-1} region corresponded to bands in the IR spectra of their respective commerical samples. The production of all three isomeric hydroxybenzoic acids (HBA) is not in agreement with a previous report.[2]

To clarify the hydroxylation mechanism, the effects of reaction component concentrations and of certain additives (buffers, complexing agents, metal ions, radical scavengers) on the yields of o-, m-, and pHBA were examined between pH 4.3 and 4.7, the region of highest yield.[2] Product yields are expressed as the total number of HBA molecules produced per NADH molecule initially present ($[HBA]_f/[NADH]_0$). The yields of hydroxybenzoic acids increased when the pH was decreased from 4.7 to 4.3, Table I, and when the $[BA]_0$ was increased from 2 to 10 mM, Table II. Changes in the citrate buffer concentration had little effect until the citrate was completely eliminated, whereupon $[HBA]_f/[NADH]_0$ decreased by a factor of 9, Table III; substitution of acetate buffer for citrate buffer resulted in a similar decline in $[HBA]_f/[NADH]_0$, Table III. The variations in pH, $[BA]_0$, and [citrate buffer] had little effect on the reaction product ratios. With variation of added $[O_2]$, a maximum product yield was obtained at 20% of saturation ($[O_2]_0$ = 260 μM), Figure 1, where $[NADH]_0/[O_2]_0$ was 1.15. pHBA/oHBA was independent of $[O_2]$, giving a ratio of (0.546 ± 0.009); mHBA/oHBA was

Table II. Dependence of Product Yields on Benzoic Acid Concentration[a]

$[BA]_0$/mM	$[HBA]_f/[NADH]_0$	relative yields oHBA	mHBA	pHBA
2.0	0.122 ± 0.012	1.00	0.808	0.526
5.0	0.170 ± 0.008	1.00	0.865	0.558
10.0	0.188 ± 0.004	1.00	0.710	0.539
		av 1.00	0.794 ± 0.037	0.541 ± 0.008

[a] Initial reactant concentrations: citrate buffer, 50 mM, pH 4.5; MP^+, 250 μM; NADH, 290 μM; air-saturated. Samples were handled as for Table I. Data are averages of two identical sets of measurements.

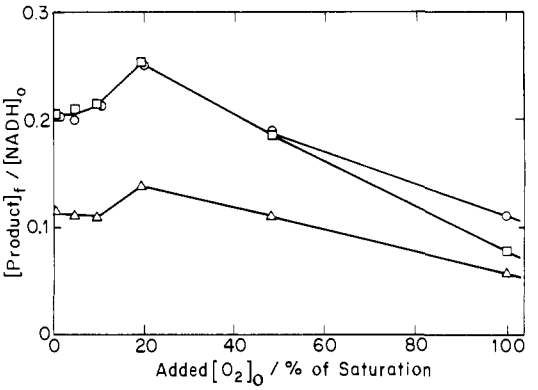


Figure 1. Dependence of product yields on added oxygen concentration. Initial reactant concentrations: citrate buffer, 50 mM, pH 4.5; BA, 10 mM; MP^+, 240 μM; NADH, 300 μM. Samples were prepared by mixing portions of O_2- and N_2-saturated solutions of buffered benzoic acid (at saturation, $[O_2]$ = 1.29 mM). Solutions (10 mL) were placed in capped reaction vessels with a total volume of ca. 11.8 mL, so that even in the experiments with 0% added oxygen, there was oxygen present. ○, □, △: yields of oHBA, mHBA, and pHBA, respectively.

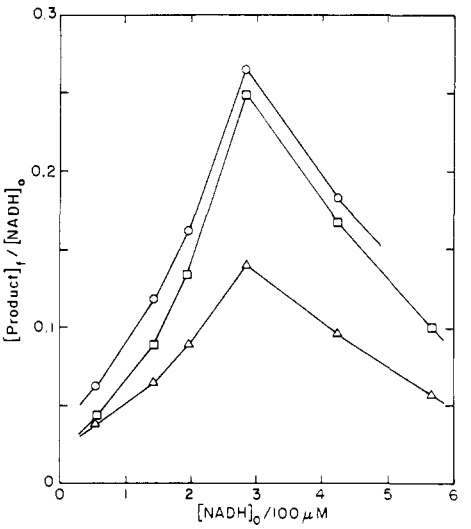


Figure 2. Dependence of the product yields on $[NADH]_0$. Initial reactant concentrations: citrate buffer, 50 mM, pH 4.5; BA, 10 mM; MP^+, 240 μM; O_2, 260 μM. Samples were prepared as described for Figure 1. ○, □. △: yields of oHBA, mHBA, and pHBA, respectively.

constant, i.e., (1.01 ± 0.01), for added $[O_2]_0 \leq 950$ μM. With added $[O_2]_0$ held constant at 260 μM, the maximum $[HBA]_f/[NADH]_0$ was obtained for $[NADH]_0$ = 280 μM, Figure 2, where $[NADH]_0/[O_2]_0$ = 1.09. pHBA/oHBA was independent of $[NADH]_0$ and was (0.538 ± 0.006); mHBA/oHBA was constant for $[NADH]_0 \geq 280$ μM, giving a ratio of (0.925 ± 0.013). With both O_2- and air-saturated samples, product yields were dependent on $[MP^+]$ and were maximized at $[MP^+] \sim 100$ μM, Figure 3. pHBA/oHBA was essentially independent of $[MP^+]$ and was

Table III. Effects of Buffers and Complexing Agents on Product Yields[a]

set	buffer	complexing agent	$[HBA]_f/[NADH]_0$	relative yields		
				oHBA	mHBA	pHBA
1[b]	50 mM citrate	none	0.193	1.00	0.869	0.535
	10 mM citrate	none	0.182	1.00	0.825	0.520
	none	none	0.020	1.00	0.865	0.594
				av 1.00	0.853 ± 0.011	0.550 ± 0.018
2[c]	50 mM citrate	none	0.386	1.00	0.948	0.505
	50 mM citrate	10 mM DETAPAC	0.294	1.00	0.880	0.478
	50 mM acetate	none	0.053	1.00	0.888	0.471
	50 mM acetate	10 mM DETAPAC	0.266	1.00	0.819	0.459
3[d]	50 mM acetate	none	0.031	1.00	0.746	0.550
	50 mM acetate	2.6 mM DETAPAC	0.125	1.00	0.772	0.513
	50 mM acetate	3.6 mM EDTA	0.042	1.00	0.852	0.564

[a] With a pK_a of 4.20,[14] benzoate itself exerts a buffering action in the pH region of the experiments so that the indicated buffers are in addition to benzoate. All experiments were at pH 4.5, with $[MP^+]_0$ = 250 μM. [b] Initial reactant concentrations: BA, 5 mM; NADH, 325 μM; air-saturated (samples were open to the air and stirred). [c] Initial reactant concentrations: BA, 10 mM; NADH 300 μM; air-saturated (samples were kept in a closed vessel during reaction); [d] Initial reactant concentrations: BA, 10 mM; NADH 260 μM; O_2, 260 μM (samples were kept in a closed vessel during reaction).

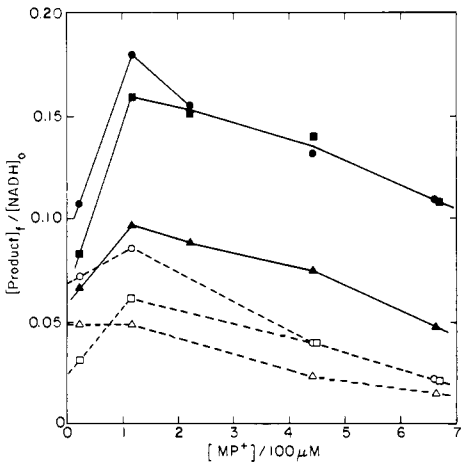


Figure 3. Effect of $[MP^+]$ on product yields at two different $[O_2]_0$. Initial reactant concentrations: citrate buffer, 50 mM, pH 4.4; BA, 10 mM; NADH, 290 μM. ○, □, △: yields of oHBA, mHBA, and pHBA, respectively, for $[O_2]_0$ = 1.29 mM. ●, ■, ▲: $[O_2]_0$ = 0.27 mM. Samples were prepared as described under Figure 1.

(0.585 ± 0.014); for $[MP^+] \geq 200$ μM, mHBA/oHBA = (0.994 ± 0.017).

In citrate-buffered samples, the addition of DETAPAC (diethylenetriaminepentaacetic acid) had little effect on reaction products, Table III; however, in acetate-buffered samples, the addition of DETAPAC dramatically increased product yields. EDTA (ethylenediaminetetraacetic acid) stimulated the acetate-buffered reaction only slightly. The addition of cyanide ion (from 96 μM to 4.8 mM) to citrate-buffered samples had no effect on product yields when the pH was carefully controlled.

The effects of the additions of complexes of Fe^{III} with citrate, acetate, DETAPAC, EDTA, and cyanide (i.e., hexacyanoferrate(III)) were determined. Addition of $FeCl_3$ to citrate-buffered solutions, producing the ferricitrate complex, increased product yields dramatically, Figure 4. The addition of complexes of Fe^{III} with DETAPAC or EDTA to acetate-buffered samples gave $[HBA]_f/[NADH]_0$ essentially equal to those obtained with ferricitrate. The behavior of the acetate-buffered system upon addition of Fe^{III}, producing ferriacetate, followed the same pattern as that of the citrate-buffered system; however, the decline in $[HBA]_f/[NADH]_0$ with increasing $[Fe^{III}]_0$ occurred at a much lower concentration in the acetate system. The addition of hexacyanoferrate(III) to citrate-buffered reaction solutions caused a substantial depression of the product yields. With $[Fe(CN)_6^{3-}]_0$ = 200 μM, $[HBA]_f/[NADH]_0$ was reduced to 35% of the value obtained in the absence of this metal complex.

The addition of Cu^{II} (as $CuSO_4$) to the citrate-buffered reaction system resulted in decreased product yields, Table IV. As $[Cu^{II}]_0$ was increased, $[HBA]_f/[NADH]_0$ *increased*, approaching the

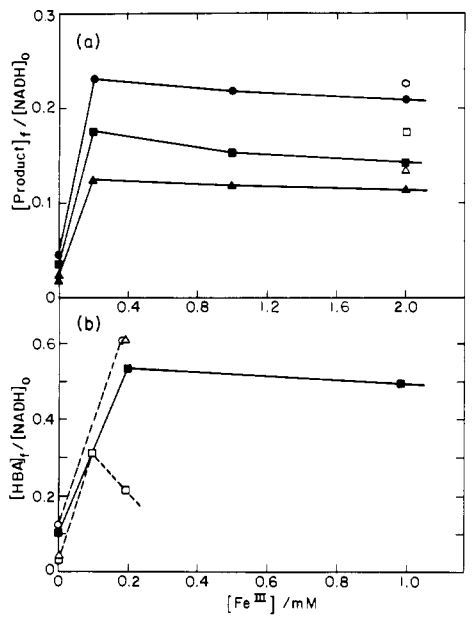


Figure 4. Effect of complexes of Fe^{III} on product yields. (a) Initial reactant concentrations: citrate buffer, 50 mM, pH 4.5; BA, 10 mM; NADH, 165 μM; O_2, 260 μM. ●, ■, ▲: yields of oHBA, mHBA, and pHBA, respectively, for $[MP^+]$ = 230 μM, ○, □, △: $[MP^+]$ = 460 μM. Samples were handled as described under Figure 1. (b) Filled symbols refer to the experiments under (a). Initial reactant concentrations were: acetate buffer, 50 mM, pH 4.5; BA, 10 mM; NADH, 260 μM; MP^+, 250 μM; O_2, 260 μM: (□) no other additives; (△) [EDTA] = 3.5 mM; (○) [DETAPAC] = 2.7 mM.

Table IV. Effect of Cu^{II} on Product Yields[a]

$[Cu^{II}]_0$/ mM	$[HBA]_f/[NADH]_0$	relative yields		
		oHBA	mHBA	pHBA
0.099	0.105	1.00	0.762	0.453
0.986	0.156	1.00	0.853	0.512
9.86	0.258	1.00	0.776	0.437
19.5	0.252	1.00	0.775	0.423
		av 1.00	0.792 ± 0.018	0.456 ± 0.017
0	0.306	1.00	0.932	0.509

[a] Initial reactant concentrations: citrate buffer, 50 mM, pH 4.5; BA, 10 mM; MP^+, 240 μM; NADH, 325 μM; air-saturated (samples were kept in a closed vessel during reaction).

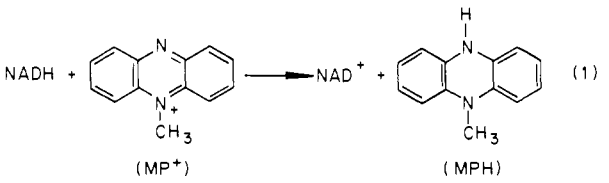
value with no added Cu^{II}; with $[Cu^{II}]_0$ = 20 mM, $[HBA]_f/[NADH]_0$ was 82% of the value obtained in the absence of Cu^{II}. Relative product yields in the presence of Cu^{II} were unaffected by $[Cu^{II}]_0$. Product yields were reduced by the addition of formate ion (HCO_2^-), Table V. The total product yields and the yields

Table V. Effect of Formate on Product Yields[a]

$[formate]_0$/mM	$[HBA]_f/[NADH]_0$	% inhibition obsd	% inhibition calcd[b]	relative yields oHBA	relative yields mHBA	relative yields pHBA
0	0.318	0	0	1.00	1.03	0.568
0.93	0.268	15.7	16	1.00	0.945	0.495
4.67	0.157	50.6	49	1.00	0.682	0.315
9.33	0.099	68.9	66	1.00	0.768	0.270

[a] Initial reactant concentrations: citrate buffer, 50 mM, pH 4.5; BA, 2.4 mM; NADH, 275 μM; MP^+, 235 μM; O_2, 260 μM. Samples were handled as described for Figure 1. [b] Calculated % inhibition ≡ fraction of HO· which would react with formate/formic acid, compared to reaction with benzoate/benzoic acid. Calculated using pK_a from ref 14, and k(HO· + benzoate/benzoic acid) = 6.0×10^9 M^{-1} s^{-1}, k(HO· + formate) = 3.5×10^9 M^{-1} s^{-1}, and k(HO· + formic acid) = 1.5×10^8 M^{-1} s^{-1} from ref 15.

Scheme I

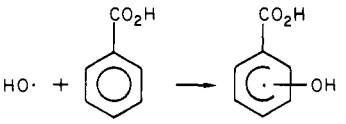


(1)

$$MPH + O_2 \xrightarrow{H^+} MP^+ + H_2O_2 \quad (2)$$

$$MPH + M^{n+} \longrightarrow MPH^+\cdot + M^{(n-1)+} \quad (3)$$

$$M^{(n-1)+} + H_2O_2 \longrightarrow M^{n+} + HO^- + HO\cdot \quad (4)$$



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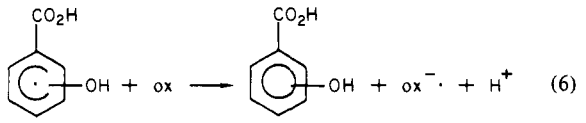


(6)

$$MP\cdot + H^+ \rightleftharpoons MPH^+\cdot \quad (7)$$

$$MP^+ + MPH \rightleftharpoons MPH^+\cdot + MP\cdot \quad (8)$$

$$MP\cdot + O_2 \longrightarrow MP^+ + O_2^-\cdot \quad (9)$$

$$O_2^-\cdot + M^{n+} \longrightarrow O_2 + M^{(n-1)+} \quad (10)$$

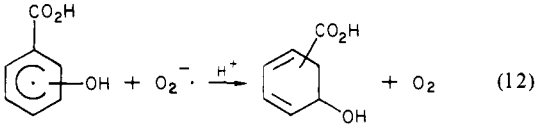
$$O_2^-\cdot + M^{(n-1)+} \xrightarrow{2H^+} H_2O_2 + M^{n+} \quad (11)$$

of mHBA and pHBA relative to oHBA declined steadily with increasing $[HCO_2^-]_0$.

Discussion

The reaction mechanism given in Scheme I is proposed for the hydroxylation of benzoic acid by the 5-methylphenazinium (MP^+)/reduced β-nicotinamide adenine dinucleotide (NADH)/O_2 system in citrate-buffered aqueous solution. $MPH^+\cdot$ and MP· denote the acid/base pair of the one-electron reduced form of MP^+; MPH denotes the two-electron reduced (and protonated) form of MP^+. The three isomeric hydroxybenzoic acids (HBA) are produced via reactions 1–6: (1) two-electron reduction of MP^+ by NADH to produce MPH; (2) direct reduction of O_2 by MPH, via hydride-ion transfer, to give H_2O_2; (3) production of hydroxyl radical (HO·) from H_2O_2 in an adventitious-metal-ion catalyzed, Fenton-type reaction driven by MPH; (4) addition of HO· to benzoic acid producing all possible isomeric hydroxycyclohexadienyl radicals (HO· adducts); and (5) oxidation of the HO· adducts to give HBA (ox in reaction 6 refers to several possible oxidants, e.g., MP^+, H_2O_2, O_2, and chelated metal ions, M^{n+} (citrate)). Reactions 7–11 are included to explain the observed results. The sole function of NADH is reduction of MP^+; this assignment is supported by experiments in which the addition of MPH (prepared externally by reduction of MP^+ with ascorbate) to oxygenated benzoate solutions resulted in the formation of hydroxylated products in the absence of NADH.[16] Whether the reduction of MP^+ by NADH proceeds via hydride-ion transfer or via two successive one-electron steps cannot be assessed from the experimental results. The role of MP^+ as a catalyst is demonstrated by the color changes occurring during the reaction period, as described in the Experimental Section.

The results implicating the hydroxyl radical as the species initiating hydroxylation in the MP^+/NADH/O_2 system are the following: the expected products of HO· reaction with benzoic acid are produced, i.e., the three isomeric HBA;[17] under conditions where oxidation of the hydroxycyclohexadienyl radicals is favored, the relative yields of HBA approach the values found for the reaction of HO· with benzoic acid under equivalent, controlled conditions;[18] and scavengers of HO· inhibit hydroxylation. The hydroxylation of aromatic species by HO· in the presence of suitable oxidants is well established.[11,19–22] Reaction of benzoic acid with HO· proceeds via addition of HO· to the aromatic ring with production of all three of the possible isomeric hydroxycyclohexadienyl radicals.[17,23] Oxidation of the radicals yields the corresponding HBA. Alternatively, the HO· adducts can dimerize,[24] eliminate water to produce a phenyl radical,[25] or be reduced to the corresponding cyclohexadiene (e.g., reaction 12).



(12)

The attack of HO· is essentially nonspecific, and oHBA, mHBA, and pHBA are produced in very nearly statistical proportions when the HO· adducts are quantitatively oxidized.[11] The relative product yields from reaction of HO· (generated radiolytically) with benzoic acid (under conditions of quantitative oxidation) are oHBA/mHBA/pHBA = 1.00/1.02/0.59 under

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(17) Under some conditions, additional products were observed in the chromatograms. A compound with the same retention time as phenol was noted in some experiments.
(18) We have examined the yields of isomeric HBA from HO· attack on benzoic acid by means of ^{60}Co γ radiolysis of N_2O-saturated aqueous solutions, using $Fe(CN)_6^{3-}$ as a radical oxidant. With the reaction conditions of Schuler and co-workers,[11] we obtained virtually identical relative yields, i.e., oHBA/mHBA/pHBA = 1.00/1.30/0.72. However, when solutions corresponding to those used in the MP^+/NADH/O_2 experiments ([BA] = 10 mM, [citrate buffer] = 50 mM, pH 4.5, $[Fe(CN)_6^{3-}]$ = 1 mM, N_2O-saturated) were irradiated, the relative isomer yields were 1.00/1.02/0.59. This ratio is substantially closer to the statistical ratio, i.e., 1.00/1.00/0.50, and may indicate a pH dependence in the positional attack of HO· on benzoic acid, related to the acid dissociation of the carboxylic group.
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(23) Addition to the ipso carbon, which results in decarboxylation and production of phenol, occurs to a limited extent.[11,22]
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our experimental conditions.[18] In the $[O_2]_0$-variation experiments, the relative product yields for $[O_2]_0 \leq 50\%$ of saturation were oHBA/mHBA/pHBA = 1.00/1.01/0.546. In the $[MP^+]$-variation experiments, the relative product yields were oHBA/mHBA/pHBA = 1.00/0.994/0.585. These ratios are remarkably close to those obtained in the radiolytic experiments.

In several sets of experiments, it was noted that when $[HBA]_f/[NADH]_0$ decreased, the yield of mHBA often decreased more rapidly than the yields of the two other HBA. In the O_2-variation experiments, $[HBA]_f/[NADH]_0$ decreased when $[O_2]_0$ was increased above 20% of saturation; pHBA/oHBA remained constant, but mHBA/oHBA declined. The decrease in $[HBA]_f/[NADH]_0$ results from the increase in the rate of reaction 2 with respect to that of reaction 3. The increase in $[O_2]$ also stimulates the production of $O_2^{-}\cdot$ (via reaction 9), which can reduce the adducts (reaction 12); the meta isomer is more rapidly attacked, leading to changes in relative yields of HBA. Similarly, in the $[NADH]_0$-variation experiments, $[HBA]_f/[NADH]_0$ decreased with decreasing $[NADH]_0$ (below the optimal concentration); pHBA/oHBA was constant, but mHBA/oHBA decreased. The declines in $[HBA]_f/[NADH]_0$ and mHBA/oHBA correlate with increasing $[O_2]$, in the manner just described. In the formate (HCO_2^-) experiments, $[HBA]_f/[NADH]_0$ declined with increasing $[HCO_2^-]_0$ owing to increased scavenging of HO· by HCO_2^-, which circumvents reaction 5. The result of this scavenging is production of the carbon dioxide radical anion.[26]

$$HCO_2^- + HO\cdot \rightarrow CO_2^{-}\cdot + H_2O \qquad (13)$$

Electron transfer from $CO_2^{-}\cdot$ to O_2 is rapid, so that in essence HO· is converted to $O_2^{-}\cdot$ by HCO_2^-. If $O_2^{-}\cdot$ were converted to H_2O_2 (via catalyzed disproportionation, reactions 10 and 11), or if $O_2^{-}\cdot$ only oxidized HO· adducts (reaction 6), changes in the relative yields of products would not be expected. However, as $[HCO_2^-]_0$ was increased, the yields of mHBA and pHBA declined more rapidly than the yield of oHBA. Since these changes can be attributed solely to the reduction of HO· adducts by $O_2^{-}\cdot$, the rate of attack of $O_2^{-}\cdot$ on the HO· adducts occurs in the order meta > para > ortho. Thus, while O_2 can oxidize HO· adducts[24,27] (producing $O_2^{-}\cdot$), it also functions effectively as a mediator in the one-electron *reduction* of the adducts.

Both citric and formic acids (and their conjugate bases) react with HO· at appreciable rates,[15] while neither reacts appreciably with $O_2^{-}\cdot$[26] (the reaction of HO· with formate is utilized in radiation chemistry to convert HO· to $O_2^{-}\cdot$[26]). We observed that increases in the ratios of citrate or formate to benzoic acid resulted in increasing inhibition of hydroxylation. With formate, the observed percent of inhibition correlates well with the calculated percent of HO· reacting with formate, Table V.

The results of several experiments demonstrate that H_2O_2 is the precursor of HO·: catalase (EC 1.11.1.6), which catalyzes the decomposition of H_2O_2, is an effective inhibitor of hydroxylation;[3] in the absence of O_2, MPH and H_2O_2 hydroxylate benzoic acid exactly as in the $MP^+/NADH/O_2$ system;[16] MPH is oxidized by O_2 to yield a precursor of HO· which is not $O_2^{-}\cdot$; and all other experimental observations can be rationalized by assuming this source of HO·. MPH is oxidized by both O_2 and H_2O_2,[13] and from the results of variation of $[O_2]$ and [NADH] on $[HBA]_f/[NADH]_0$, it is clear that both reactions are necessary for hydroxylation. O_2 is required for production of H_2O_2 (reaction 2); however, the reaction of MPH with H_2O_2 is inhibited by high $[O_2]$ (competition between reactions 2 and 3), and lower product yields are obtained when the ratio of NADH to O_2 decreases. The optimal balance between reactions 2 and 3 was reached at $[NADH]_0/[O_2]_0 \sim 1.1$, where the highest $[HBA]_f/[NADH]_0$ were obtained.

H_2O_2 is reduced by MPH to produce HO·. It is not possible to determine, a priori, whether this reduction occurs directly (reaction 14), or indirectly in a catalyzed Fenton-type reaction

$$MPH + H_2O_2 \rightarrow MPH^{+}\cdot + HO^- + HO\cdot \qquad (14)$$

(reactions 3 and 4). In citrate-buffered experiments where $[NADH]_0/[O_2]_0$ was well below the optimal value, addition of Fe^{III} resulted in a dramatic increase in $[HBA]_f/[NADH]_0$.[28] This effect can be rationalized as follows. In the presence of excess O_2, reaction 2 is favored over reactions 3 and 14; ample H_2O_2 is produced, but MPH is not available to reduce it. The addition of Fe^{III} accelerates reactions 3 and 4 ($M^{n+} = Fe^{III}$), thereby diverting MPH to the reduction of H_2O_2 (just as occurs when $[O_2]$ is reduced). Complexes of iron with citrate, DETAPAC, and EDTA all work equally well as catalysts for reactions 3 and 4.[29] Thus, it is clear that the catalyzed decomposition does occur. In the absence of added iron, citrate and DETAPAC are equally efficient in stimulating the hydroxylation process; $[HBA]_f/[NADH]_0$ can be as large as values obtained with added iron. Alternatively, acetate and EDTA provide little stimulation of reaction. These results are rationalized if, in the absence of added metal ions, H_2O_2 decomposition occurs via reactions 3 and 4, supported by adventitious metal ions, and not via reaction 14 (analysis by atomic absorption spectroscopy of several of the reagents employed showed iron contamination).[30] Kinetic data in a subsequent investigation support this conclusion.[16]

The H_2O_2 utilized in the production of HO· arises from the reduction of O_2 by MPH. Two paths are available: one-electron reduction to give $O_2^{-}\cdot$ which in turn undergoes catalyzed disproportionation to H_2O_2 and O_2 (reactions 10 and 11),[31] or a concerted, two-electron reduction (hydride-ion transfer) producing H_2O_2 directly (reaction 2). The Cu^{II}/Cu^{I} pair is an effective catalyst for $O_2^{-}\cdot$ disproportionation, as reactions 10′ and 11′ are

$$O_2^{-}\cdot + Cu^{II} \rightarrow O_2 + Cu^{I} \qquad (10')$$

$$O_2^{-}\cdot + Cu^{I} \xrightarrow{2H^+} H_2O_2 + Cu^{II} \qquad (11')$$

both rapid ($k_{10'} = 8 \times 10^9\ M^{-1}\ s^{-1}$; $k_{11'} \sim 10^{10}\ M^{-1}\ s^{-1}$);[32] however, if Cu^{II} were present at sufficiently high concentrations, reaction 11′ virtually would be suppressed[33] by the scavenging of nearly all $O_2^{-}\cdot$ in reaction 10′, and the production of H_2O_2 would be suppressed. As a result, the addition of high $[Cu^{II}]$ provides a test for the contribution of $O_2^{-}\cdot$ to the production of H_2O_2: if $O_2^{-}\cdot$ were a major source of H_2O_2, the addition of high $[Cu^{II}]$ would substantially reduce $[HBA]_f/[NADH]_0$. In the present experiments with $[Cu^{II}] = 20$ mM, $[HBA]_f/[NADH]_0$ was 82% of the value obtained in the absence of Cu^{II}; under the experimental conditions employed, >88% of any $O_2^{-}\cdot$ present would be converted to O_2 via reaction 10′.[33] Thus, $O_2^{-}\cdot$ is at best only a minor source of H_2O_2 in this system, and it is clear that most of the oxidation of MPH by O_2 proceeds via direct production of H_2O_2. This result

(25) At sufficently low radical concentrations (with inadequare oxidant present), the bimolecular dimerization reaction gives way to the elimination of H_2O from the HO· adduct with production of a phenyl radical (this is equivalent to abstraction of an H atom by the HO· radical). Dehydration of hydroxycyclohexadienyl radicals in acid solution occurs via an acid-catalyzed loss of hydroxide, with production of a radical–cation intermediate, followed by deprotonation to the phenyl radical (see, e.g., ref 22 and Sehested, K.; Holcman, J.; Hart, E. J. *J. Phys. Chem.* **1977**, *81*, 1363–7).

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(28) Although the metal chelates increased $[HBA]_f/[NADH]_0$, the relative yield of mHBA remained low, perhaps indicating that these chelates are not able to oxidize the HO· adducts (particularly the meta adduct). This is quite possible, since a major effect of chelation is often reduction of the metal-ion redox potential. In support of this idea, at higher concentrations of ferricitrate, $[HBA]_f/[NADH]_0$ was depressed slightly, the meta isomer being affected especially; a doubling of $[MP^+]$ restored $[HBA]_f/[NADH]_0$ to the maximum value via, one may presume, more efficient oxidation of HO· adducts, particularly of the sensitive meta isomer.

(29) If the redox potential of the chelated metal is sufficiently high, it can decrease $[HBA]_f/[NADH]_0$ by competitively oxidizing NADH or MPH, as was observed with ferricyanide and ferriacetate. With ferriacetate, the stimulation of hydroxylation at *low* metal ion concentrations was apparent.

(30) The iron content of reagents was determined by atomic absorption spectroscopy (Mellon Institute, Analytical Services; error limit of ±15 ppm): benzoic acid, 32 ppm, sodium acetate, 63 ppm; sodium citrate, 126 ppm; citric acid, 0 ppm; EDTA, 127 ppm; and DETAPAC, 63 ppm.

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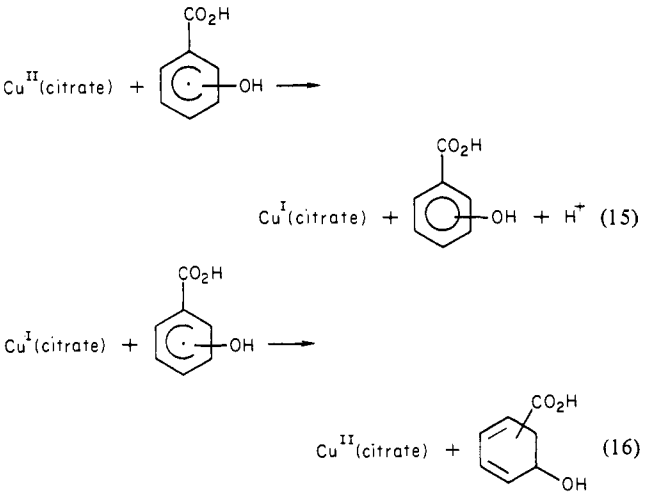

is consistent with the observation of Halliwell[3] that superoxide dismutase did not affect hydroxylation yields.

Walling[24] has reported that ***high*** concentrations of Cu^{II} will oxidize a substantial portion of the HO· adducts of several aromatics. Bhatia and Schuler[20] have shown that copper ions can both oxidize (Cu^{II}) and reduce (Cu^{I}) the HO· adducts of benzene. Thus, in the present experiments, reactions 15 and 16 must be considered. As $[Cu^{II}]_0$ is decreased, the ratio of Cu^{I}(citrate) to Cu^{II}(citrate) will increase so that the frequency of reaction 16 relative to reaction 15 will increase. The overall result of this catalysis of the disproportionation of the HO· adducts by the copper ions is that $[HBA]_f/[NADH]_0$ will decrease as $[Cu^{II}]_0$ is decreased. This is exactly the trend observed. It is probable that the lowered value of $[HBA]_f/[NADH]_0$ obtained at the highest $[Cu^{II}]_0$ (when compared with no copper added) results from this effect and not from scavenging of $O_2^{-}\cdot$, discussed in the previous paragraph.

Increasing $[MP^+]$ results in a shift of equilibrium 8 to the right, so that $[MP\cdot]$ and $[MPH^+\cdot]$ increase. If oxidation of the reduced MP^+ species occurred only via reactions 2 and 3, $[HBA]_f/[NADH]_0$ would be independent of $[MP^+]$; however, it was observed in both air- and oxygen-saturated experiments that increasing $[MP^+]$ above a critical level resulted in decreases in $[HBA]_f/[NADH]_0$. A clue to the reaction mode is the effect of $[O_2]$ on the decreases in the product yields: in air- and O_2-saturated experiments, $[HBA]_f/[NADH]_0$ obtained at the maximum $[MP^+]$ were 51.6 and 29.3%, respectively, of the maximum yields obtained. Thus, O_2 plays a role in the depression of the product yields, probably via reaction 9.[34] In essence, the reaction of MPH with O_2 (in competition with reaction of MPH with H_2O_2) is facilitated by the shift of equilibrium 8 to the right, so that the effect of increasing $[MP^+]$ on $[HBA]_f/[NADH]_0$ is the same as that of increasing $[O_2]$ (reaction 12 is prevented by the high $[MP^+]$). The decrease in $[HBA]_f/[NADH]_0$ as $[MP^+]$ is taken below the critical level (ca. 100 μM) demonstrates the importance of MP^+ in the oxidation of the HO· adducts (reaction 6): as $[MP^+]$ is decreased, the nonproductive reactions compete more effectively with reaction 6, so that $[HBA]_f/[NADH]_0$ declines and relative product yields change.

Finally, if the hydroxylation of benzoic acid proceeded via the most direct steps (reactions 1–6), at most, one molecule of HBA would be produced for each molecule of NADH consumed ($[HBA]_f/[NADH]_0 = 1$):

$$NADH + BA + O_2 \xrightarrow{H^+} NAD^+ + HBA + H_2O \quad (17)$$

However, under no experimental conditions was this theoretical limit attained; the maximum value found for $[HBA]_f/[NADH]_0$ was 0.65.

Conclusions

In the hydroxylation of benzoic acid by the 5-methylphenazinium (MP^+)/reduced β-nicotinamide adenine dinucleotide (NADH)/O_2 system, myriad reactive species and free radicals are produced. A number of the reactions are coupled so that a change in one reaction parameter results in a cascade of changes in the hydroxylation system. The main conclusions regarding the hydroxylation mechanism are that (i) the species initiating hydroxylation is the hydroxyl radical, (ii) hydroxyl radicals are produced by a one-electron reduction of H_2O_2 (a Fenton-type reaction driven by MPH), (iii) H_2O_2 arises almost exclusively from a concerted, two-electron reduction of O_2 by MPH, and (iv) superoxide radicals are produced to only a very limited extent by the $MP^+/NADH/O_2$ system.

From the reactions seen in hydroxylation of benzoic acid, it is apparent that whenever MP^+, molecular oxygen, and a reasonably strong reducing agent (e.g., NADH or ascorbate) are present in aqueous solution, one can obtain hydrogen peroxide and hydroxyl radicals, as well as MPH which is a good reducing agent capable of one- and two-electron reduction. Thus, in the study of dopamine-β-hydroxylase by Liu, Shen, and Ganong,[9] where $MP^+/NADH/O_2$ was utilized as a purported $O_2^{-}\cdot$ generator, it is probable that a species such as MPH was serving as a reducing agent for the enzyme copper ions—simply substituting for ascorbate. These observations may have wide applicability to a large number of biological studies which employ the 5-methylphenazinium cation as an electron carrier.

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Registry No. Benzoic acid, 65-85-0; 5-methylphenazinium methyl sulfate, 299-11-6; formate, 71-47-6; reduced NADH, 58-68-4; DETAPAC, 67-43-6; EDTA, 60-00-4; O_2, 7782-44-7; Cu^{II}, 15158-11-9; Fe^{III}, 20074-52-6.

(33) The percent of $O_2^{-}\cdot$ which disappears via reaction 11′ is

$$\% \text{ reaction } 11' = 100[Cu^I]k_{11'}/([Cu^I]k_{11'} + ([Cu^{II}]_0 - [Cu^I])k_{10'})$$

A limiting value can be computed using a $[Cu^I]_{max}$ obtained by assuming that all $O_2^{-}\cdot$ enter reaction 10′, all HO· adducts are oxidized by Cu^{II} (reaction 15), and Cu^I is not reoxidized:

$$[Cu^I]_{max} = [O_2^{-}\cdot]_{max} + [\text{HO}\cdot \text{ adduct}]_{max}$$

The maximum possible yield of HO· adducts would be obtained if each $O_2^{-}\cdot$ were reduced to H_2O_2 (e.g., via reaction 11′), and each H_2O_2 yielded two hydroxyl radicals:

$$[\text{HO}\cdot \text{ adduct}]_{max} = 2[H_2O_2]_{max} = 2[O_2^{-}\cdot]_{max}$$

If all MPH reacts with O_2 via one-electron transfer, the maximum, total $[O_2^{-}\cdot]$ would be:

$$[O_2^{-}\cdot]_{max} = 2[NADH]_0$$

so that

$$[Cu^I]_{max} = 3[O_2^{-}\cdot]_{max} = 6[NADH]_0$$

and

$$(\% \text{ reaction } 11')_{max} = 600([NADH]_0/[Cu^{II}]_0)k_{11'}/k_{10'}$$

For the experimental values $[Cu^{II}]_0 = 19.5$ mM and $[NADH]_0 = 325$ μM; (% reaction 11′)$_{max}$ is 12%. Since $[Cu^I]$ is clearly substantially less than $[Cu^I]_{max}$, the percent of $O_2^{-}\cdot$ which react with Cu^I to give H_2O_2 is well below 12%.

(34) $MPH^+\cdot$ reacts *very* slowly[13] with O_2; however, MP· will be present at the pH of these experiments, and MP· has a redox potential substantially lower than that of $MPH^+\cdot$.[13,35]

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