Oxidation of 4-Methylcatechol: Implications for the Oxidation of Catecholamines

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ABSTRACT: At alkaline pH, 4-methylcatechol oxidizes more rapidly than the related catecholamines: dopamine, norepinephrine, and epinephrine. This oxidation is not inhibited by superoxide dismutase or catalase, indicating that O_2 itself is the oxidant, but the reduction potential of $O_2/O_2^{-\bullet}$ is too low for it to oxidize 4-methylcatechol directly. Instead, O_2 oxidizes the 4-methylcatechol semiquinone, which is formed by comproportionation of 4-methylcatechol and its *o*-quinone. Aniline reacts very quickly with the *o*-quinone and thus stops the comproportionation reaction that oxidizes 4-methylcatechol to the semiquinone. Oxidation of 4-methylcatechol then requires superoxide, and in the presence of aniline, oxidation of 4-methylcatechol by O_2 is inhibited by superoxide dismutase. When catecholamines oxidize, the side chain amine inserts into the catechol *o*-quinone, forming a bicyclic compound. By eliminating the quinone, this ring closure prevents comproportionation and the consequent oxidation of catecholamines by O_2. It also prevents reaction of the quinone with other compounds and the formation of potentially toxic products.

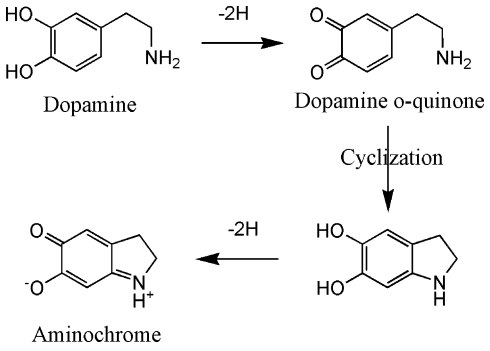
The oxidation of 4-methylcatechol (**1** in Scheme 2) has attracted attention for two reasons. First, the synthesis of the trihydroxybenzene (topaquinone) cofactor of copper amine oxidases is thought to proceed from tyrosine through a catechol intermediate (*1*−*4*). Second, the oxidation of the catecholamine dopamine may be a contributing factor in the death of dopaminergic neurons, leading to Parkinson's disease (*5*, *6*). Despite the attention given to this general reaction, the mechanism of catechol oxidation remains surprisingly controversial.

The mechanism by which molecular oxygen oxidizes catecholamines to the *o*-quinone is unsettled. Superoxide oxidizes catecholamines (*7*), suggesting a series of single-equivalent steps leading to the semiquinone and then to the quinone. It has also been argued that catechols react directly with molecular oxygen to form a hydroperoxide which then eliminates H_2O_2 to yield the *o*-quinone (*2*, *3*).

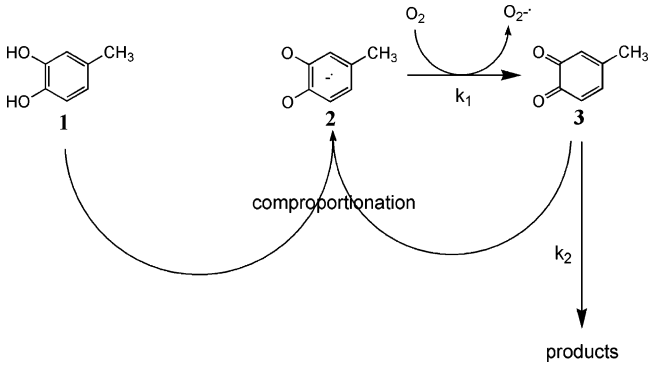
The quinone is susceptible to nucleophilic attack by amines and thiols. When catecholamines oxidize to the *o*-quinone, the side chain amine inserts into the ring forming a bicyclic compound (*8*−*10*). In the case of dopamine (Scheme 1), this process forms aminochrome, which polymerizes with other compounds to form neuromelanin (*11*−*13*). In the case of epinephrine, this product is adrenochrome. A simple assay for superoxide measures the level of formation of the strongly absorbing adrenochrome following the oxidation of epinephrine by superoxide (*7*, *14*).

In this study, we have examined the oxidation of 4-methylcatechol and 3,4-dihydroxyphenylacetic acid (DOPAC)[1] by O_2. Lacking the amine side chain, these compounds do not cyclize upon oxidation, so alternative fates of the *o*-quinone may be examined. Aniline, which rapidly attacks the *o*-quinone, provides a convenient probe. We have used aniline to determine the lifetime of the *o*-quinone in water and to show that the *o*-quinone participates in the oxidation of catechols by O_2.

Scheme 1: Oxidation Products of Dopamine



Scheme 2: Oxidation of 4-Methylcatechol in Water



EXPERIMENTAL PROCEDURES

All experiments were performed at 22 °C in 0.2 M potassium phosphate buffer (pH 8.0) containing 10 μM

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[1] Abbreviations: DOPAC, 3,4-dihydroxyphenylacetic acid; EDTA, ethylenediaminetetraacetic acid; 4-MeCat, 4-methylcatechol; SOD, superoxide dismutase.

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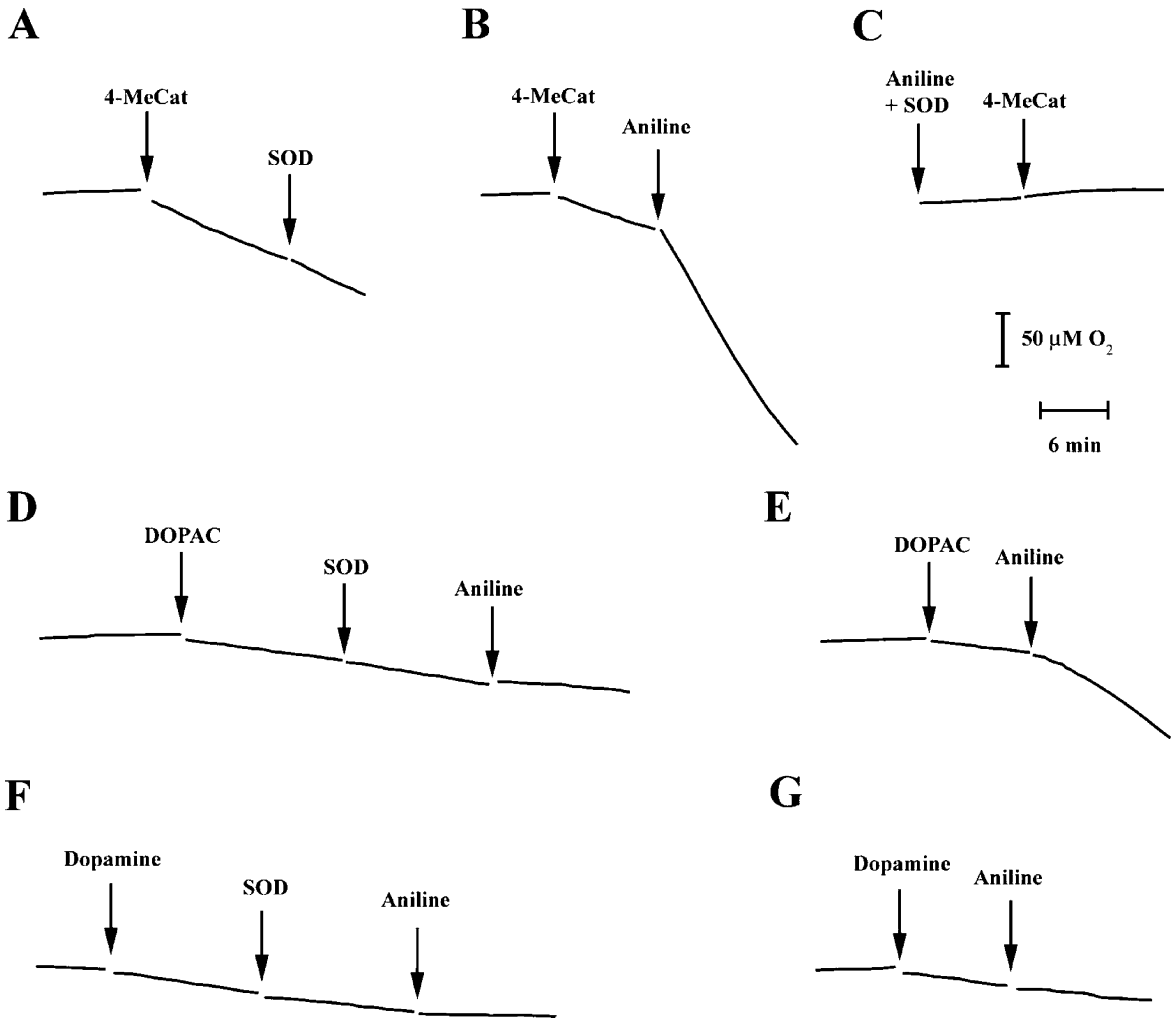


FIGURE 1: O_2 reduction by catechols at pH 8. Catechols [final concentration of 4-methylcatechol (4-MeCat), DOPAC, or dopamine of 2.3 mM], aniline (final concentration of 9.2 mM), and superoxide dismutase (37 units/mL) were added at the indicated times.

EDTA. The pH was chosen as a compromise between physiological pH and a more alkaline pH which supports faster rates of oxidation. Oxygen consumption (Figure 1) was assessed using a Clark-type oxygen electrode. Catechols were added to 4 mL of buffer containing additions as indicated. The concentration of O_2 was calibrated using glucose and glucose oxidase. Spectrophotometry was performed using a Shimadzu UV160U spectrophotometer.

Cyclic voltammograms were recorded on a BAS 100 W electrochemical analyzer equipped with a glassy carbon working electrode, a Ag/AgCl reference electrode, and a platinum wire auxiliary electrode. Sweep rates were 0.1 V/s. The glassy carbon electrode was polished with alumina and cleaned via sonication between each recording. Prior to the data being recorded, argon was bubbled through the solution to purge dissolved O_2. Cyclic voltammograms were also recorded in the buffer solution alone, and this background current was subtracted from all traces shown. Potentials are expressed relative to the standard hydrogen electrode.

Reduction potentials for 4-methylcatechol were calculated from values measured at pH 6.9 by Deakin et al. (*15*). Values measured relative to the saturated sodium calomel electrode were converted to the standard hydrogen electrode reference by adding 0.235 V. The value for the *o*-quinone/semiquinone potential is independent of pH above pH 6, so the value of 0.190 V applies at pH 8. The semiquinone/methylcatechol potential at pH 8 (0.370 V) was calculated using the equation and p*K* values given by Deakin et al. (*15*). Then, at 22 °C, the equilibrium constant (K_{eq}) for disproportionation and/or comproportionation of the 4-methylcatechol species equals $\exp[(0.190\ \text{V} - 0.370\ \text{V})(F/RT)] = 8.4 \times 10^{-4}$.

4-Methyl-5-anilino-*o*-benzoquinone (**6**) was prepared by mixing 0.5 g of 4-methylcatechol, 6.6 g of $K_3Fe(CN)_6$, 1.0 g of $KHCO_3$, and 0.7 mL of aniline in 200 mL of H_2O. After 15 min at 22 °C, the red product was extracted into chloroform. The solvent was removed by evaporation and the product redissolved in ethanol at a concentration of 52 mM. The concentration was determined from the absorption at 490 nm using an extinction coefficient of 3419 M^{-1} cm^{-1}. Purity was confirmed by thin-layer chromatography. Cyclic voltammetry also confirmed the presence of a single electrochemically active product with a potential matching that shown in Figure 4.

4-Methylcatechol (Aldrich) was recrystallized from hot toluene (*15*). Aniline was added as a 0.2 M aqueous stock solution. DOPAC was obtained from Aldrich, and superoxide dismutase, catalase, adrenochrome, and dopamine were from Sigma Chemical Co.

RESULTS

Although dopamine oxidation has attracted considerable attention, dopamine is oxidized by O_2 at pH 8 more slowly

Table 1: Rates of O_2 Reduction[a]

	without catalase	with catalase
no additions	0.0367 ± 0.0059 μM/s (3)	0.0202 ± 0.0012 μM/s (3)
with SOD	0.0467 ± 0.0065 μM/s (3)	0.0282 ± 0.0023 μM/s (3)
with aniline	0.2404 ± 0.0147 μM/s (5)	0.1347 ± 0.0198 μM/s (4)
with aniline and SOD	0.0127 ± 0.0039 μM/s (3)	–

[a] Rates were determined from slopes of traces as shown in Figure 1. Aniline (9.2 mM), catalase (92 μg/mL), and superoxide dismutase (37 units/mL) were added as indicated. Reduction of O_2 was initiated by adding 4-methylcatechol (final concentration of 2.3 mM). Values are the average ± standard deviation of the number of measurements given in parentheses.

than the related 4-methylcatechol (Figure 1A,F). To clarify the reasons for this, we examined the oxidation of 4-methylcatechol. The rate of oxygen reduction by 4-methylcatechol is slightly stimulated by superoxide dismutase (Table 1), indicating that 4-methylcatechol is oxidized by O_2 itself and not by superoxide. Catalase reduces the rate by nearly 50% (Table 1), confirming that H_2O_2 is the final product of O_2 reduction. DOPAC (Figure 1D,E) oxidizes more slowly than 4-methylcatechol, but the rate responds similarly to SOD and aniline.

Aniline provides a very useful tool for studying the oxidation of catechols. Because it attacks *o*-quinones very rapidly (*16*), aniline can be used to eliminate the *o*-quinones formed by the oxidation of 4-methylcatechol and DOPAC. This mimics the effect of cyclization in catecholamine oxidation and makes it possible to assess the role of the *o*-quinone in the oxidation process. Interestingly, superoxide dismutase inhibits O_2 reduction in the presence of aniline (Figure 1C) but not when aniline is absent (Figure 1A), so superoxide is required to oxidize 4-methylcatechol only when aniline is present and reacting with the *o*-quinone.

When aniline reacts with the catechol quinone, this nucleophilic attack yields a reduced compound (compound **4**, Scheme 3), which then oxidizes rapidly to **6**. Because the oxidized aniline adduct (**6**) absorbs strongly at 490 nm, its formation may be followed spectrophotometrically (Figure 2). Superoxide dismutase inhibits the appearance of compound **6**, consistent with its inhibition of O_2 reduction by 4-methylcatechol in the presence of aniline.

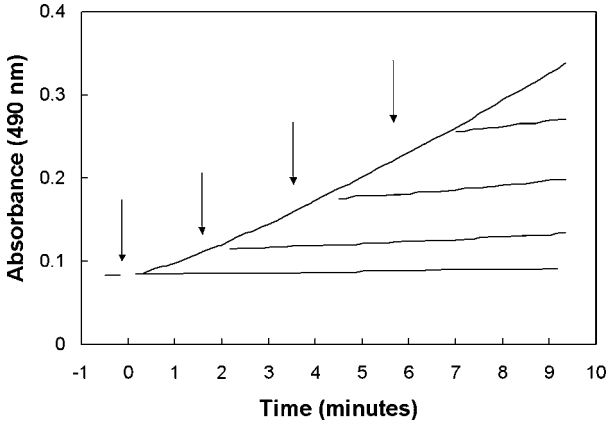
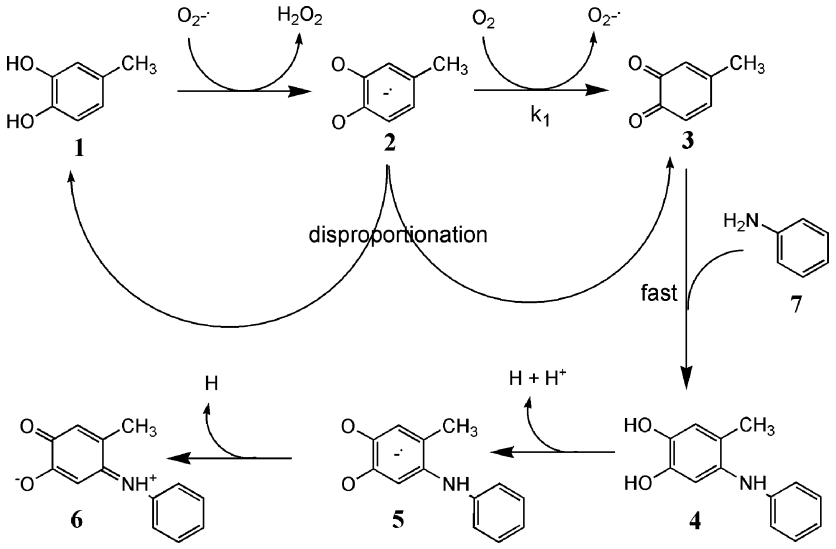


FIGURE 2: Formation of the 4-methylcatechol−aniline adduct. The absorbance of 2 mL of 0.2 M phosphate buffer and 10 μM EDTA (pH 8.0) containing catalase (0.18 mg/mL) and aniline (9.1 mM) was recorded at 490 nm. At time zero, 4-methylcatechol (final concentration of 2.3 mM) was added. Superoxide dismutase (120 units) was added at the times indicated by arrows.

The oxidized aniline adduct (**6**) may be formed quickly by oxidizing 4-methylcatechol with ferricyanide and then adding aniline. The rate of appearance of the 490 nm absorbance following aniline addition is not proportional to either the aniline concentration or the concentration of 4-methylcatechol, indicating that the rate-limiting step is not the formation of the 4-methylcatechol−aniline adduct (**4**) but its subsequent oxidation to **6**. Consequently, no attempt was made to determine a rate constant for addition of aniline to the *o*-quinone.

In the absence of aniline, the *o*-quinone disappears slowly with a half-time of ~16 min at pH 8. The product(s) absorbs at 480 nm (*2*, *3*, *17*, *18*). Therefore, there is a slow increase in absorbance following oxidation of the catechol with ferricyanide in the absence of aniline (Figure 3). The absorbance was measured at 490 nm to avoid interference from the ferricyanide absorbance at 418 nm. Because aniline reacts with the 4-methyl-*o*-quinone much more rapidly, adding aniline at any time after 4-methylcatechol oxidation leads to a rapid increase in absorbance corresponding to the amount of *o*-quinone remaining. Thus, the disappearance of the *o*-quinone may also be followed as the loss of aniline

Scheme 3: Oxidation of 4-Methylcatechol in the Presence of Aniline



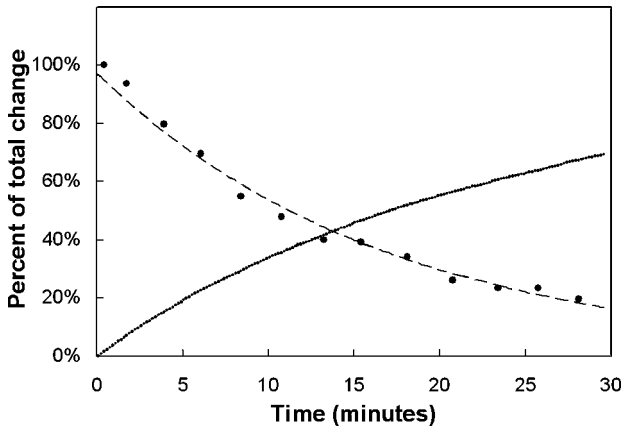


FIGURE 3: Lifetime of 4-methyl-*o*-quinone. Formation of the hydroxyquinone anion was monitored by mixing 4-methylcatechol (final concentration of 1.14 mM) with 9.1 mM $K_3Fe(CN)_6$ in 0.2 M potassium phosphate and 10 μM EDTA (pH 8.0) and monitoring the absorbance at 490 nm (—). The disappearance of the catechol quinone was monitored by mixing 4-methylcatechol with ferricyanide in the same way and then periodically diluting 0.2 mL aliquots into 2 mL of 0.2 M potassium phosphate and 10 μM EDTA (pH 8.0) and measuring the change in absorbance upon addition of 100 μL of 0.2 M aniline. The increase in absorbance at 490 nm caused by aniline is plotted vs time of aniline addition (●). The dashed line is an exponential fit to these points.

Table 2: Rate Constants for the Disappearance of *o*-Quinone[a]

	4-methylcatechol	DOPAC
absorbance at 490 nm	$(7.0 \pm 0.8) \times 10^{-4}\ s^{-1}$ (3)	$(7.5 \pm 0.7) \times 10^{-3}\ s^{-1}$ (3)
loss of aniline reactivity	$(7.2 \pm 0.5) \times 10^{-4}\ s^{-1}$ (2)	–

[a] Rate constants were determined from the experiment shown in Figure 3 following oxidation of 4-methylcatechol or DOPAC. The loss of aniline reactivity and the increase in hydroxyquinone absorbance at 490 nm were fit to exponential functions to obtain rate constants. Values are the average ± standard deviation of the number of measurements given in parentheses.

reactivity (Figure 3). The rate constant measured by the two methods is comparable (Table 2). The rate of *o*-quinone loss is much faster for the *o*-quinone of DOPAC (half-time of ~1.5 min) than for that of 4-methylcatechol (Table 2).

Cyclic voltammetry may be used to characterize the electrochemistry of catechols and their adducts. The cyclic voltammogram of 4-methylcatechol shows a typical reversible pattern with a reduction potential of 0.28 V (Figure 4). Dopamine, by contrast, displays a diminished reduction peak because the oxidized product is removed by the side chain cyclization reaction. A reduction peak for the bicyclic product aminochrome is observed at a considerably lower potential. In the presence of aniline, the cyclic voltammogram of 4-methylcatechol becomes more like that of dopamine. The reduction wave disappears as the quinone is removed by reaction with aniline. Peaks for the aniline adduct appear at lower potentials, although not as low as that of aminochrome.

In the absence of SOD, aniline accelerates the oxidation of 4-methylcatechol (Figure 1B) and DOPAC (Figure 1E). Therefore, the aniline adduct itself was tested to see whether it affects the oxidation of 4-methylcatechol. In fact, addition of 4-methyl-5-anilino-*o*-quinone (**6**) does increase the rate of this oxidation (Figure 5). By contrast, adrenochrome, the

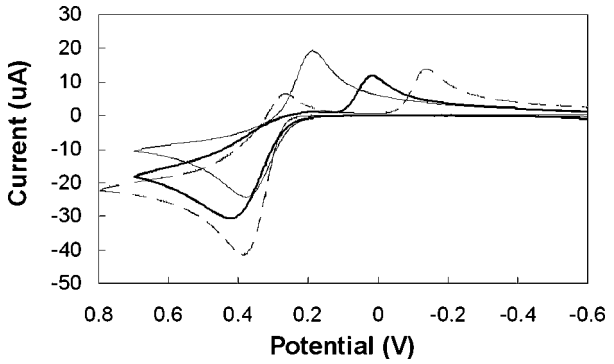


FIGURE 4: Cyclic voltammograms of catechols: (thin solid line) 1 mM 4-methylcatechol in 0.1 M potassium phosphate and 10 μM EDTA (pH 8.0), (thick solid line) 1 mM 4-methylcatechol in the presence of 0.5 mM aniline, and (dashed line) 1 mM dopamine.

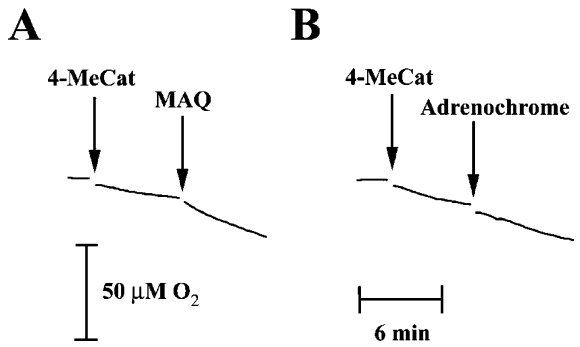


FIGURE 5: Acceleration of 4-methylcatechol oxidation by 4-methyl-5-anilino-*o*-quinone but not by adrenochrome. O_2 reduction was recorded with 2.3 mM 4-methylcatechol (4-MeCat), 250 μM 4-methyl-5-anilino-*o*-quinone (MAQ), and 250 μM adrenochrome added at the indicated times.

analogue of aminochrome formed by cyclization of the *o*-quinone of epinephrine, has no effect.

DISCUSSION

The reduction potential of catechols is too high for them to reduce O_2 to superoxide at an appreciable rate. (The midpoint potential of 4-methylcatechol/semiquinone is 0.370 V at pH 8 as described in Experimental Procedures.) Thus, reaction with O_2 might occur in one of two ways. (1) Catechol (**1**) may react with superoxide to yield the catechol semiquinone (**2**) and H_2O_2. (2) The catechol semiquinone (**2**) may react with O_2 to yield the catechol quinone (**3**) and superoxide. A third possibility, that O_2 may add to the catechol (**1**) forming a hydroperoxide which then eliminates H_2O_2 to yield the quinone (**3**), has been suggested (*2*, *3*). This mechanism was derived from studies of the autoxidation of 4-methylcatechol in the presence of Cu(II) and does not seem to apply under the conditions described here. Because it does not involve either superoxide or the catechol quinone, that mechanism cannot account for the observed effects of SOD and aniline.

Given the considerations described above, the oxidation of 4-methylcatechol in the absence and presence of aniline can be explained by Schemes 2 and 3, respectively. In the absence of aniline, catechol (**1**) and its quinone (**3**) comproportionate to yield the semiquinone (**2**). Comproportionation of catechols and their *o*-quinones has been reported for 3,5-di-*tert*-butylpyrocatechol (*19*) and for estrogen catechols (*20*, *21*). The semiquinone produced by comproportionation then

reacts with O_2 to yield the quinone, which is slowly converted to other products.

In the presence of aniline (**7** in Scheme 3), the catechol quinone is converted rapidly to **4**, so the quinone is not available for the comproportionation reaction. In this case, therefore, superoxide is needed to oxidize the catechol (**1**) to the semiquinone (**2**). The semiquinone may then be oxidized to the quinone (**3**) by O_2. This latter reaction yields superoxide required for the first step. Compound **4** rapidly oxidizes to **6**, yielding additional superoxide.

These reaction pathways may be analyzed quantitatively to confirm that the observed rates are consistent with the rate constants of the individual reactions. Rates measured in the presence of catalase (Table 1) have been used for the analysis so any effects of H_2O_2 may be ignored. In the presence of catalase, one molecule of O_2 is reduced to H_2O for each molecule of 4-methylcatechol converted to oxidized adduct (e.g., **6**). Consequently, the measured rates for both O_2 consumption and adduct formation are the same.

At the steady state in the absence of aniline, the rate of catechol quinone disappearance (k_2[**3**]) equals the rate (V) of O_2 consumption. Therefore, the quinone concentration is given by

$$[\mathbf{3}] = V/k_2 \quad (1)$$

Given the value of k_2 given in Table 2 and the rates measured in Table 1, the steady-state quinone concentration in these experiments is then 40 μM in the presence of SOD and 29 μM in its absence. At these concentrations, the rate of comproportionation or disproportionation is fast (*19*), so the catechol (**1**), *o*-quinone (**3**), and semiquinone (**2**) species equilibrate, and their concentrations are related by

$$K_{eq} = [\mathbf{2}]^2/([\mathbf{1}][\mathbf{3}]) \quad (2)$$

K_{eq}, calculated from reduction potentials as described in Experimental Procedures, is 8.4×10^{-4}. The 4-methylcatechol concentration ([**1**]) is 2.5 mM. Given the values for [**3**] calculated above, the semiquinone concentrations are 9.2 μM in the presence of SOD and 7.8 μM in its absence.

In the presence of SOD, the rate of oxidation of **2** by O_2 is limiting, and this step must occur twice for each 4-methylcatechol oxidized to the quinone. Therefore

$$2V = k_1[O_2][\mathbf{2}] \quad (3)$$

Concentrations of 250 μM for O_2 and 9.2 μM for **2** imply a value of 25 M^{-1} s^{-1} for k_1. This value may be compared to the rate constant for an outer-sphere electron transfer reaction calculated using the cross relation from Marcus theory (*22*, *23*):

$$k_{12} = (k_{11}k_{22}K_{12}f_{12})^{1/2} \quad (4)$$

The equilibrium constant ($K_{12} = 1.05 \times 10^{-6}$) may be calculated from reduction potentials: -0.16 V for $O_2/O_2^{-\bullet}$ (*24*) and 0.19 V for **3**/**2** (*15*). The self-exchange rate constant for $O_2/O_2^{-\bullet}$ (k_{11}) is 450 M^{-1} s^{-1} (*25*). The self-exchange rate constant for **3**/**2** (k_{22}) is likely to be $\sim 6 \times 10^7$ M^{-1} s^{-1}, the value determined for benzoquinone (*26*). The collision factor ($f_{12} = 0.17$) may be calculated from the values given above (*23*). The theoretical rate constant as calculated using eq 4 is then 70 M^{-1} s^{-1}, in excellent agreement with the observed value of 25 M^{-1} s^{-1}. Therefore, oxidation of 4-methylcatechol is consistent with a mechanism in which the catechol and the *o*-quinone comproportionate and the resulting semiquinone is oxidized by O_2 via an outer-sphere electron transfer. Aniline, via removal of the quinone, prevents comproportionation, so the catechol then requires superoxide to oxidize to the semiquinone. Superoxide is generated by reduction of O_2 by the semiquinone and by the aniline adduct. Aniline accelerates 4-methylcatechol and DOPAC oxidation both because its adduct generates $O_2^{-\bullet}$ when it oxidizes and because it removes the quinone which would otherwise be reduced back to the semiquinone by $O_2^{-\bullet}$.

The aniline adduct (4-methyl-5-anilino-*o*-quinone) is also able to accelerate the oxidation of 4-methylcatechol by oxidizing it directly (Figure 5). With a reduction potential of $\sim$0.028 V, this adduct oxidizes 4-methylcatechol at a slow rate, despite the unfavorable potential difference and the fact that aniline itself is not present to remove the *o*-quinone product. This happens because the adduct is very rapidly reoxidized by O_2. Adrenochrome, which has a lower reduction potential, does not oxidize 4-methylcatechol quickly enough to affect the rate significantly. Thus, the lower potential of adrenochrome and the bicyclic products of other catecholamines is important in minimizing catecholamine oxidation mediated by the oxidation products themselves.

This mechanism highlights two important consequences of the cyclization reaction of the dopamine quinone. The elimination of the *o*-quinone prevents autocatalytic oxidation of dopamine driven by comproportionation of dopamine and the dopamine *o*-quinone. It also prevents the dopamine *o*-quinone from reacting with other substances to form toxic products. The significance of the latter has also been noted by Tse et al. (*16*).

The oxidation of DOPAC is slow compared to the oxidation of 4-methylcatechol, even though DOPAC cannot form a bicyclic structure like dopamine. This is probably because it has a lower steady-state quinone concentration as a consequence of its faster rate of decay. When eqs 1−3 are rearranged, it can be shown that

$$V = (k_1^2[O_2]^2K_{eq}[\mathbf{1}])/4k_2 \quad (5)$$

Thus, if other factors are equal, the rate of oxidation (V) will be inversely proportional to the rate constant for *o*-quinone degradation (k_2). DOPAC, with a rate constant 10-fold greater than that of 4-methylcatechol (Table 2), would be expected to oxidize one-tenth as quickly.

This mechanism of catechol oxidation also rationalizes the fact that superoxide oxidizes epinephrine to adrenochrome but is less effective at converting dopamine to aminochrome (Scheme 1). The conversion of epinephrine to adrenochrome has been exploited as an assay for superoxide and superoxide dismutase (*7*, *14*), but dopamine does not work nearly as well in this assay. This may be understood by recognizing that the cyclization of the dopamine quinone to form aminochrome is in competition with the reduction of the quinone by superoxide. The rate constant for the reaction of the 4-methylcatechol quinone with superoxide may be calculated from k_1 and the equilibrium constant for the reaction. As described earlier, the equilibrium constant calculated from reduction potentials is 1.05×10^{-6} and k_1

is 25 M^{-1} s^{-1}. This implies a rate constant of 2.4×10^7 M^{-1} s^{-1} for the reverse reaction. The rate constant for the cyclization of the dopamine quinone is 0.5 s^{-1} at pH 8 (*27*). If we assume that k_{-1} has a value similar to that determined for 4-methylcatechol, then the dopamine quinone will be reduced by superoxide faster than it will form the bicyclic product if the superoxide concentration is greater than ~20 nM. In superoxide assays, the superoxide concentration will typically be much larger than this, and the yield of the aminochrome product will therefore be very small. Epinephrine has a cyclization rate constant several orders of magnitude larger than that of dopamine (*10*), so the superoxide concentration would need to approach 1 μM before the back reaction becomes a factor. Thus, the back reaction with superoxide may prevent the dopamine quinone from forming aminochrome but does not affect the formation of adrenochrome from the epinephrine quinone simply because of the great difference in cyclization rates.

In summary, the catechol quinone promotes catechol oxidation by participating in comproportionation to yield the semiquinone, which is more readily oxidized by O_2. Dopamine oxidation is slow because ring closure removes the dopamine quinone preventing comproportionation. DOPAC oxidation is slow because addition of H_2O removes the catechol quinone. Understanding the rates of these reactions and the factors that affect them may illuminate conditions that tip the balance from normal catecholamine metabolism to pathological states leading to Parkinson's disease.

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