The Oxidation of Catecholamines and 6-Hydroxydopamine by Molecular Oxygen: Effect of Ascorbate

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Dedicated to Professor Dieter Seebach on the occasion of his 60th birthday

Catecholamines, 6-Hydroxydopamine, Ascorbate, Autoxidation, Redox Cycling

Comparative kinetic studies on the oxidation of catecholamines (CA) (dopamine (DA), epinephrine (EP), norepinephrine (NEP)) serving as a neuromediator in the sympathetic nervous system, 3,4-dihydroxyphenylalanine (DOPA) and 6-hydroxydopamine (6-OHDA), a well-known neurotoxic agent, were performed in the presence of ascorbate ($AscH^-$) in 50 mM phosphate buffer, pH 7.40, at 37 °C by using a Clark electrode, EPR and the absorption spectroscopy. The oxidation of CA and DOPA alone was found to be a self-accelerating process, with quinone products (Q) acting as autocatalysts. The rate of oxygen consumption (R_{OX}) increased with time and reached a steady-state level. A starting value of R_{OX} increased in the order: EP < DOPA ≈ NEP << DA << 6-OHDA, whereas a steady-state value of R_{OX} changed in the order: DOPA < DA < NEP << EP << 6-OHDA. The changes in R_{OX} with time were found to correlate with the resistance of primary Q to the intramolecular cyclization.

The effect of $AscH^-$ on CA oxidation depended dramatically on whether $AscH^-$ was added to non-oxidized or preoxidized CA. Added to non-oxidized CA and DOPA, $AscH^-$ inhibited their oxidation (but not that of 6-OHDA). For the case of DA, a pronounced lag period was observed by both a Clark electrode and spectrophotometrically. The addition of $AscH^-$ to preoxidized CA, DOPA and 6-OHDA induced an increase in R_{OX} and a steady-state concentration of the ascorbyl radical. The kinetic behaviour of the systems was determined by two major factors: 1) $AscH^-$ suppressed the formation of Q, a catalyst for CA oxidation, most likely due to the reaction of $AscH^-$ with the semiquinone formed from CA; 2) Q derived both from CA and 6-OHDA catalyzed $AscH^-$ oxidation. The elevated cytotoxicity of 6-OHDA was found to be in part caused by the condition that 6-OHDA oxidation was not inhibited by $AscH^-$ and by the high efficiency of 6-OHDA as a redox cycling agent in combination with $AscH^-$. These observations explain the very pronounced and prolonged cytotoxicity of 6-OHDA even at low concentrations that increases at elevated concentrations of $AscH^-$.

Introduction

Catecholamines (CA)* dopamine (DA), epinephrine (EP) and norepinephrine (NEP) as well as 3,4-dihydroxyphenylalanine (DOPA)** a biogenic precursor of CA, are readily oxidized by molecular oxygen at physiological pH and temperature with the formation of active forms of oxygen (Bindoli *at al.,* 1992; Graham *et al.*, 1978; Afanas'ev, 1989, and references therein). CAs are potentially toxic and the oxidation of CA in vivo is frequently considered as a triggering mechanism for some neuropathologies, including schizophrenia

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* *Abbreviatons:* CA, catecholamine; DA, dopamine; EP, epinephrine; NEP, norepinephrine; DOPA, 3,4-dihydroxyphenylalanine; 6-OHDA, 6-hydroxydopamine; Q, a quinone product of CA or 6-OHDA oxidation; $Q^{\bullet -}$, a semiquinone; $AscH^-$, $Asc^{\bullet -}$, the ascorbyl radical; EDTA, ethylenediaminetetraacetate; R_{OX}, a rate of oxygen consumption; t_{ind}, induction period; Δt, period of CA preoxidation.

** Although DOPA does not formally belong to the CAs, in the following no distinction is made between DOPA and genuine CA.

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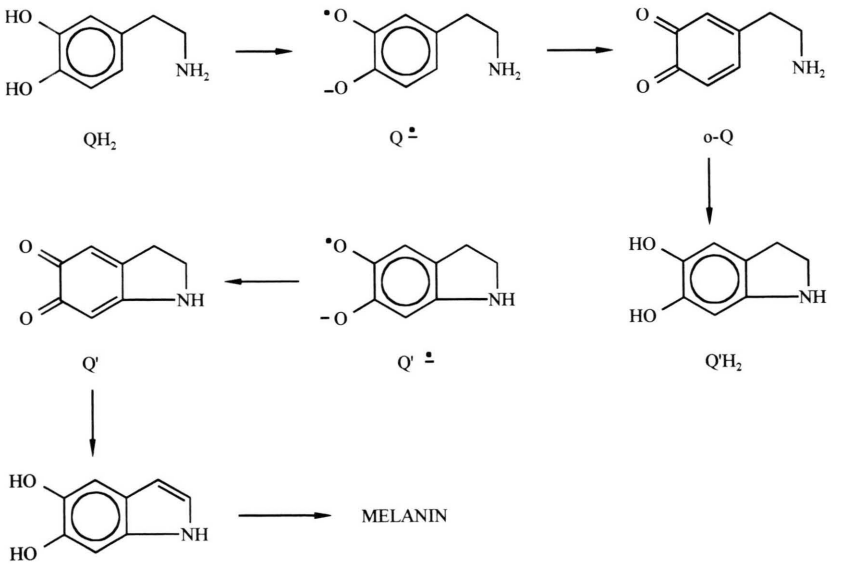
and Parkinson's disease (Lake and Ziegler, 1985; Bindoli *at al.,* 1992; Jenner, 1994). CA concentration is very high in some cells, nevertheless it is common for the normal status of the body. This suggests a very effective mechanism to inhibit CA oxidation *in vivo* that remains hitherto an open question. 6-OHDA, a hydroxylated derivative of DA, showing outstanding oxidizability, exerts a pronounced selective toxicity upon nerve cells including neuroblastoma (Giunta *et al.,* 1991; Sachs and Jonsson, 1975). This led to the suggestion to apply 6-OHDA for 'chemical surgery via targeted free radical cell killing' (Borg *et al.*,1978), particularly, as an agent for neuroblastoma treatment (Reynolds *et al.*, 1982; Bruchelt *et al.*, 1991).

Although the oxidation of CA and 6-OHDA by molecular oxygen is broadly discussed in the literature (Graham *et al.,* 1978; Graham, 1978; Gee and Davison, 1989; Köhle *et al.*, 1995), the pathway and kinetics of these processes are known only in little detail. A plausible sequence of products formed during CA oxidative transformation is given in Scheme I for the example of DA. The process most likely begins with the formation of *o*-semiquinone anion-radical ($Q^{\bullet -}$), followed by its transformation into a primary *o*-quinone (Q) *via* the reaction of $Q^{\bullet -}$ with oxygen or/and $Q^{\bullet -}$ disproportionation. The intramolecular cyclization of primary *o*-Q results in the formation a hydroxylated product ($Q'H_2$) and induces a cascade of oxidative reactions resulting in the formation of melanin, an insoluble polymer pigment. Nerve cells are rich not only in CA but also in ascorbate ($AscH^-$) (Pileblad *et al.*, 1988). Therefore many studies have been focused on the oxidation of CA and 6-OHDA in the combination with $AscH^-$. $AscH^-$ was reported to enhance the rate of oxygen consumption (R_{OX}) during the oxidation of CA and 6-OHDA (Borg *et al.*, 1978; Pileblad *et al.*, 1988) as well as their toxicity (Reynolds *et al.*, 1982; Bruchelt *et al.*, 1991, Fornstedt and Carlsson, 1991). However the molecular mechanisms of these effects are unknown.

In this study the kinetics of the autoxidation of CA and 6-OHDA both alone and in combination with $AscH^-$ were studied in more detail. It was found that a temporal increase in the rate of the oxidation is a fundamental feature of the process under consideration, depending on the accumulation of Q. It is suggested that rather complex regularities of the oxidation of CA and 6-OHDA in combination with $AscH^-$ are controlled by two main factors: 1) quinoid products of oxidative transformation both of CA and 6-OHDA catalyze $AscH^-$ oxidation; 2) $AscH^-$ inhibits the oxidation of endogenous CAs (but not that of 6-OHDA) due to the reaction of $AscH^-$ with $Q^{\bullet -}$. The difference in the thermodynamic properties of $Q^{\bullet -}$ may account for the difference in kinetic behavior of CA and 6-OHDA in the presence of $AscH^-$.



Scheme I. Proposed oxidative pathway for catecholamine by the example of DA.

Materials and Methods

DA, EP, NEP, DOPA, 6-OHDA (Sigma), ascorbic acid (Fluka) were used as received. Sodium phosphates, NaH_2PO_4 and Na_2HPO_4, of highest grade were purchased from Merck. All other materials were of highest available grade. Aqueous solutions were prepared with twice-distilled water. 50 mM solutions of NaH_2PO_4 and Na_2HPO_4, used for buffer preparation, were freed of traces of transition metals using Chelex-100 resin (Bio-Rad), following Buettner, 1988. The oxidation was studied in 50 mM phosphate buffer, pH 7.40±0.02, at 37.0±0.1 °C, unless otherwise specified. Generally, CA was added as solid, whereas 6-OHDA and $AscH^-$ were added as acid stock solutions, pH≈3.

The kinetics of oxygen uptake were studied with a Clark-type electrode (Yellow Springs Instrument Co. Model 5300 Biological Oxygen Monitor) under vigorous stirring by a magnetic agitator. Prior to adding active components, 3 ml buffer was stirred in the reaction chamber for 10 min to reach the temperature, oxygen concentration, and electrochemical equilibrium. The rate of oxygen uptake (R_{OX}) was calculated from the slope of $[O_2]$ traces. As R_{OX} was expected to depend on $[O_2]$, R_{OX} was determined over a narrow range of $[O_2]$ from 100 to 80% of the starting value, about 0.2 mM. When $[O_2]$ dropped below this level, monitoring $[O_2]$ was suspended and a plunger with a Clark electrode was lifted from the tested solution at steady stirring for a few minutes to restore the starting level of $[O_2]$, then the plunger was immersed into solution again and monitoring $[O_2]$ was resumed. A kinetic curve of oxygen uptake could be reconstructed by the integration of plot of R_{OX} vs. time.

A steady-state concentration of $Asc^{\bullet -}$ was determined by EPR with a Varian E 12 spectrometer operating at 9.25 GHz and equipped by a temperature controller. Instrument settings were: a modulation frequency, 12.5 kHz; a microwave power, 5 mW; a modulation amplitude, 0.63 G. Other details of $[Asc^{\bullet -}]$ determination have been reported elsewhere (Roginsky and Stegmann, 1994).

Temporary changes in UV-Vis absorption spectra during the oxidation of DA were determined with an M40 UV-Vis spectrophotometer (Carl Zeiss Jena) with vigorous stirring by a magnetic agitator.

The values presented below are the means of two to five runs (root-mean-square errors are given). The typical deviations of measurements did not exceed ±10% for R_{OX} and ±15% for $[Asc^{\bullet -}]$.

Results

The oxidation of CA without ascorbate

The autoxidation of CAs occurred with a substantial self-acceleration (Fig. 1A), R_{OX} increased progressively with time and achieved a steady-

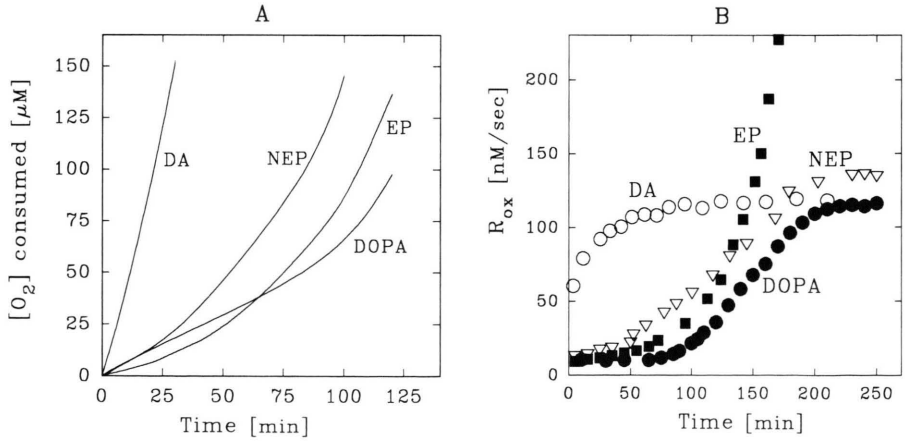


Fig. 1. The kinetics of oxygen consumption during the oxidation of 10 mM CAs. **A** – $[O_2]$ traces in the initial stage. **B** – temporal changes in a rate of oxidation, R_{OX}. With 10 mM EP the steady-state value of R_{OX} was too high to be measured; with 2.5 mM EP it was found to be 205 nM/s.

state value, $(R_{OX})_{SS}$, differed for various compounds (Fig. 1B, Table I). The starting values of R_{OX}, $(R_{OX})_0$, increase in the order of EP < DOPA ≈ NEP << DA (Table I) what is reasonably consistent with a report of Graham *et al.*, 1978. As for $(R_{OX})_{SS}$, the order of CAs was substantially different from that of $(R_{OX})_0$: EP >> NEP > DA > DOPA (Table I). Both $(R_{OX})_0$ and $(R_{OX})_{SS}$ varied nearly directly with the starting concentration of CA as exemplified by EP in the legend to Table I. As mentioned in the Introduction and shown in Scheme I, the autoxidation of CAs does not stop at the stage of the formation of a primary Q. Secondary and succeeding hydroxylated products are expected to be involved in the oxidative transformation as well (Bindoli *et al.*, 1992; Graham *et al.*, 1978; Graham, 1978). This is supported by the fact that the ultimate number of oxygen molecules consumed per one molecule of CA determined at CA oxidation under more severe conditions (elevated pH or photoactivation with methylene blue) was as much as 3–4 for DA, EP and NEP (not shown).

Table I. The starting ($(R_{OX})_0$) and steady-state ($(R_{OX})_{SS}$) rates of oxygen consumption during the oxidation of 10 mM CA and 6-OHDA.

Substrate	$(R_{OX})_0$ [nM/s]	$(R_{OX})_{SS}$ [nM/s]
EP	6.5±3.5	(820)[a]
DOPA	11±4	115 ±10
NEP	13±5	135±7
DA	75±6	120±8
6-OHDA	$1.6\pm0.2\cdot10^5$ [b]	–

Note: [a] – when 10 mM EP oxidized, $(R_{OX})_{SS}$ was too high to be measured directly. $(R_{OX})_{SS}$ was found to be as much as 45 nM/s, 80 nM/s and 205 nM/s when EP concentration was 0.5 mM, 1 mM and 2.5 mM respectively. The value given in parentheses is $(R_{OX})_{SS}$ for 10 mM EP extrapolated from the above data assuming that $(R_{OX})_{SS}$ is directly with [EP]; [b] – the value is recalculated from that determined for 50 μM 6-OHDA (800 nM/s) under the assumption that $(R_{OX})_0$ is proportional to [6-OHDA].

The self-accelerating character of CA oxidation suggests a catalytic effect by oxidation products, quinoid compounds being the most likely autocatalysts. Fig. 2A demonstrates that several biologically significant quinones, even at rather low concentrations, induce a marked acceleration of CA oxidation. This was shown for adrenochrome, a product of EP oxidation, for quinoid compounds formed by the autoxidation of 6-OHDA as well as for 2,3-dimethoxy-5-methyl-1,4-benzoquinone. The complex character of traces 3 and 4 in Fig. 2A was likely due to two quinoid compounds, one added and the other formed by EP oxidation, participating in the catalysis. As the first was consumed in the course of the process, the second accumulated.

Since iron has been suggested as a catalyst of CA oxidation, the catalytic effect of iron on the oxidation of EP was compared with that of quinones (Fig. 2B). 20 μM Fe had no effect on EP oxidation (traces 1 and 2) while ethylenediaminetet-

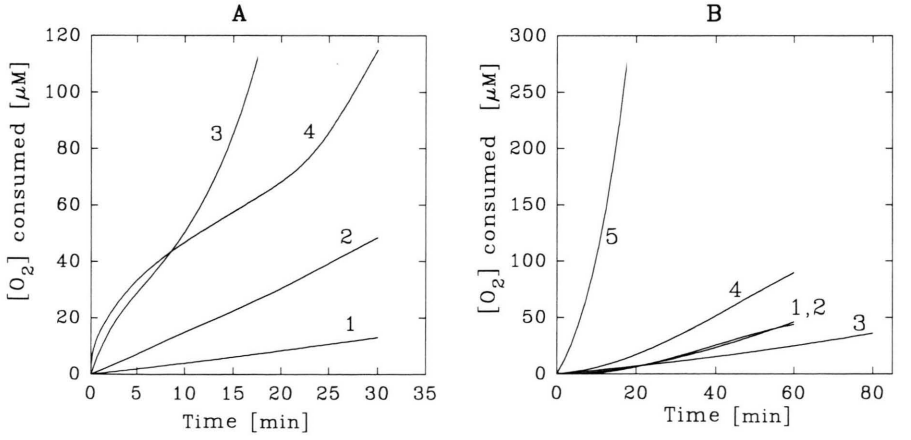


Fig. 2. Effects of additives on the kinetics of the oxidation of 10 mM EP. **A** – effects of quinones: 1 – no additives; 2 – adding 50 μM adrenochrome; 3 – adding 10 μM 2,3-dimethoxy-5-methyl-1,4-benzoquinone; 4 – adding 50 μM preoxidized 6-OHDA. **B** – effects of iron and iron chelators: 1 – no additives; 2 – adding 20 μM $FeSO_4$; 3 – adding 0.2 mM desferrioxamine; 4 – adding 0.2 mM EDTA; 5 – adding 20 μM $FeSO_4$ + 0.2 mM EDTA.

raacetate (EDTA), the chelating agent which is known to enhance catalytic activity of iron (Buettner, 1989; Roginsky and Stegmann, 1994), showed only a moderate accelerating effect (trace 4). This agrees with finding of Pileblad *et al.* (1988), that diethylenetriaminepentaacetate, another iron chelating agent, did not show any effect on DA autoxidation. The combination of 20 μM Fe with EDTA caused a dramatic increase in the rate of EP oxidation (trace 5). R_{OX} increased with time again, apparently due to a catalytic effect of the oxidative products accumulated. Similar observations were obtained with NEP and DA (not shown). In conclusion, iron causes a catalytic effect on CA oxidation when activated by EDTA. The absence of a pronounced catalytic action of EDTA without added Fe suggests that the tested specimen of CAs did not contain substantial admixtures of iron. Consequently, the catalytic capability of iron may be neglected compared to that of quinoid oxidative products.

The Oxidation of CA and 6-OHDA in Combination with Ascorbate

The effects of $AscH^-$ on CA oxidation depend dramatically on whether $AscH^-$ is added to CA from the very beginning or to preoxidized CA as previously reported for DA (Pileblad *et al.*, 1988).

Ascorbate is added to non-oxidized CA

The oxidation of DA in the presence of $AscH^-$ showed a pronounced induction period observed both by oxygen uptake (Fig. 3A) and by accumulation of colored quinone products (Fig. 3B). During the main part of the induction period R_{OX} decreased with time most likely because of the reduction of [$AscH^-$] due to oxidation. This observation along with the absence of quinoid colored products typical of DA oxidation gives evidence that oxidation of $AscH^-$ rather than that of DA occurred during the induction period. After completion of an induction period, which likely corresponded to the moment of the exhaustion of $AscH^-$, both oxygen consumption (Fig. 3A) and the accumulation of colored products (Fig. 3B) proceeded at the rate typical of the oxidation of DA alone. The induction period (t_{ind}) increased with [$AscH^-$] and decreased with added Fe(EDTA), a catalyst of CA oxidation (Table II).

$AscH^-$ inhibited the oxidation of EP, NEP and DOPA as well. However, in contrast to the DA-$AscH^-$ system, other CAs did not exhibit a pronounced induction period. After a rather long period, when R_{OX} decreased with time, similar to that during the oxidation of the DA- $AscH^-$ system, bending of the [O_2] trace was observed. Thereafter R_{OX} increased with time as observed during the oxidation of CA alone (data not shown). Consequently, $AscH^-$ exerts inhibition on the oxidation of all the CAs studied. Some differ-

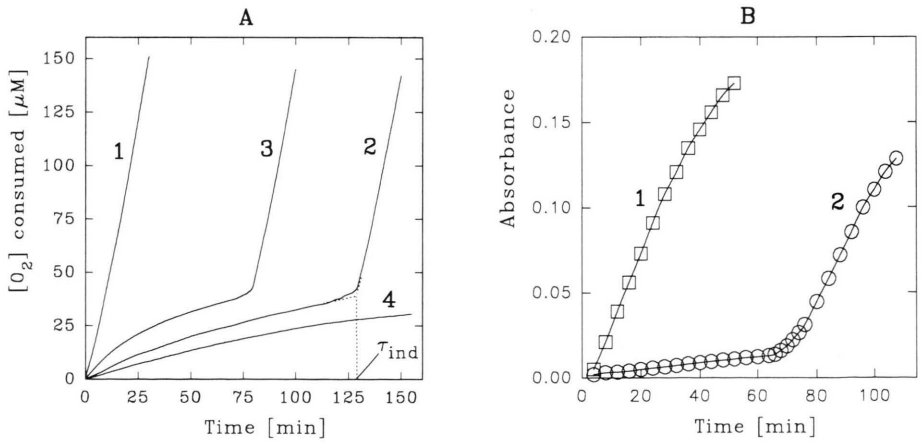


Fig. 3. Inhibiting effects of $AscH^-$ on the oxidation of 10 mM DA as studied by a Clark electrode (**A**) and spectrophotometrically spectra at 450 nm (**B**). **A** – trace 1 – DA without additives; trace 2–50 μM $AscH^-$ added to non-oxidized DA; trace 3–50 μM $AscH^-$ added to DA preoxidized during 10 min; trace 4–50 μM $AscH^-$ alone. **B** – plot 1 – DA alone; plot 2 – in the presence of 25 μM $AscH^-$.

Table II. The duration of an induction period, t_{ind}, during the oxidation of 10 mM in the presence of $AscH^-$, $FeSO_4$ and 50 μM EDTA as determined with a Clark electrode[a].

$[AscH^-]$ [μM]	[Fe] [μM]	t_{ind} [min]
25	0	74[b,c]
50	0	128[b]
50	0.25	59
50	1.0	26
100	1.0	44
200	1.0	80
1000	0	>500[b]

Note: [a] – the procedure of t_{ind} determination may be derived from Fig. 3; [b] – without adding EDTA; [c] – determined spectrophotometrically as shown in Fig. 4.

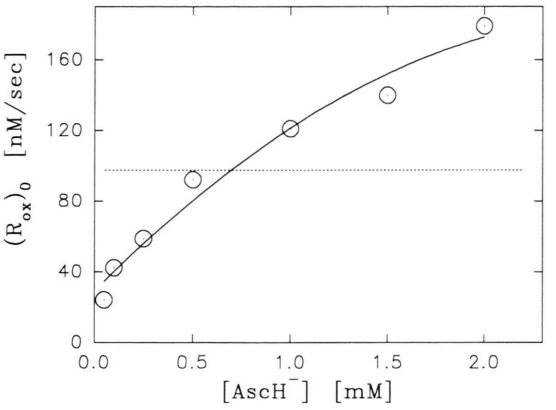


Fig. 4. Starting values of the rate of oxygen consumption, R_{OX}, when various concentrations of $AscH^-$ were added to 10 mM DA preoxidized for 10 min. The dotted line shows the value of R_{OX} determined for the oxidation of DA alone.

ences in the kinetic behavior between DA and other CAs are obviously caused by the differences in the kinetics of the oxidation of individual CAs.

Ascorbate is added to a preoxidized CA

When the concentration of $AscH^-$ added was rather low and the duration of DA preoxidation, Δt, was not too long, the DA- $AscH^-$ system again showed a pronounced induction period (Fig. 3A, trace 3). However, it was shorter than that observed when $AscH^-$ was added to non-oxidized DA. The longer Δt, the less pronounced was the induction period. A value of R_{OX} determined immediately after adding $AscH^-$, $(R_{OX})_0$, increased with Δt. For example, when 50 μM $AscH^-$ was added to 10 mM preoxidized DA, $(R_{OX})_0$ increased from 5.0 n*M*/s at Δt = 0 to 24 nM/s at Δt = 10 min. and 58 nM/s at Δt = 70 min. At constant Δt $(R_{OX})_0$ increased with $[AscH^-]$. The addition of $AscH^-$ may result, depending on $[AscH^-]$, in either a decrease or increase in $(R_{OX})_0$ compared with that measured for the oxidation of DA alone. Certainly, a critical concentration of added $AscH^-$ which does not change $(R_{OX})_0$, about 0.65 mM $AscH^-$ for the system presented in Fig. 4, depends on Δt and [DA]. Similar observations were made when $AscH^-$ was added to preoxidized EP, NEP and DOPA (not shown).

The intricate kinetic regularities observed when $AscH^-$ was added to preoxidized CA may be explained by the simultaneous action of two factors: 1) inhibition of $AscH^-$ on CA oxidation; 2) accelerating (catalytic) effect of Q, the products of CA oxidative transformation, on $AscH^-$ oxidation.

The first effect is evidently caused by the reduction of $Q^{\bullet -}$ by $AscH^-$

$$Q + AscH^- + H^+ \Longleftrightarrow QH_2 + Asc^{\bullet -} \qquad (1)$$

(see below for more detail). The second effect is likely due to the interaction of Q with $AscH^-$:

$$Q + AscH^- \longrightarrow Q^{\bullet -} + Asc^{\bullet -} + H^+ \qquad (2)$$

that was suggested in (O'Brien, 1991). EPR technique yielded additional arguments for a catalytic role of Q *via* reaction (2). When 10 mM CA was oxidized alone at pH 7.4 at 37 °C, no EPR signal was observed. The oxidation of $AscH^-$ is accompanied by the appearance of a characteristic EPR signal from $Asc^{\bullet -}$ (two main lines with a splitting of 1.81±0.02 G (Roginsky and Stegmann, 1994 and references therein)). The concentration of $Asc^{\bullet -}$ was reported to correlate with a rate of $AscH^-$ oxidation (Roginsky and Stegmann, 1994; Buettner and Jurkiewicz, 1993). When $AscH^-$ was added to 'fresh', non-preoxidized CA, the concentration of $Asc^{\bullet -}$ was slightly lower than that without CA (Table III). If $AscH^-$ was added to preoxidized CA, $[Asc^{\bullet -}]$ increased in comparison to that in the absence of CA. The effect increased with Δt (Table III). The observation that an increase in $[Asc^{\bullet -}]$ takes place only when $AscH^-$ is added to a preoxidized CA confirms the idea that it is Q catalyzing $AscH^-$ oxidation.

Table III. Steady-state concentrations of $Asc^{\bullet -}$ determined after adding 1 mM $AscH^-$ into 10 mM preoxidized CA (Δt – a period of the preoxidation).

	[$Asc^{\bullet -}$] [nM]		
Δt [min]	DA	EP	NEP
0	43	71	79
15	160	120	nd
30	180	210	nd
45	220	nd	120
90	200	360	200
120	nd	370	210

Note: without CA added a value of [$Asc^{\bullet -}$] was as much as 105±12 nM; nd – not determined.

The oxidation of 6-OHDA in the presence of ascorbate

The oxidation of 6-OHDA occurred incomparably faster than that of CA and almost completed within about 2 min. (Fig. 5, trace 1). When the oxidation of 6-OHDA was observed in combination with $AscH^-$ (trace 2 in Fig. 5), R_{OX} again decreased dramatically with time. Then came a stationary mode (post-oxidation), when the oxidation occurred at nearly constant R_{OX} that was visibly higher than R_{OX} during the oxidation of $AscH^-$ alone (trace 3 in Fig. 5). Similar [O_2] traces were documented at all the tested concentrations of 6-OHDA (5–50 μM) and $AscH^-$ (0.05–1.0 mM). When $AscH^-$ was added to preoxidized 6-OHDA, R_{OX} remained nearly constant for a few minutes (trace 4 in Fig. 5). The latter value did not differ from R_{OX} measured during the period of post-oxidation, when $AscH^-$ was added prior to 6-OHDA (trace 2 in Fig. 5). R_{OX} during the period of post-oxidation increased with [6-OHDA]. Co-oxidation of $AscH^-$ with 6-OHDA was accompanied by an increase in [$Asc^{\bullet -}$] compared to oxidation of $AscH^-$ alone (Fig. 6). The accumulation of Q during 6-OHDA oxidation occurred at 21 °C slower than at 37 °C and it was possible to observe the growth of [$Asc^{\bullet -}$] during a few minutes (Fig. 6). A maximum value of [$Asc^{\bullet -}$] did not depend on whether $AscH^-$ was added to 'fresh' or preoxidized 6-OHDA (Fig. 6). These observations suggest that reaction (2) with the participation of accumulated Q rather than reaction (1) between $Q^{\bullet -}$ and $AscH^-$ is responsible for $Asc^{\bullet -}$ formation. Consequently, those products of 6-OHDA oxidation, along with oxidative products formed from CAs, catalyze $AscH^-$ oxidation. Unlike CA, the oxidation of 6-OHDA apparently was not inhibited by $AscH^-$.

Discussion

The self-acceleration that is most likely caused by the accumulation of quinoid products is a general feature of CA and 6-OHDA oxidation. This was previously observed with EP (Misra and Fridovich, 1972). A self-accelerating character of the autoxidation is typical of various hydroquinones including 1,4-hydroquinone (Eyer, 1991) and 1,4-hydroxynaphthalines (Bandy *et al.*, 1990). The ability of Q to catalyze the oxidation of organic

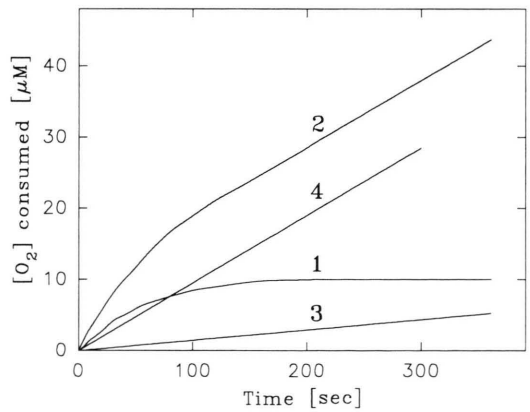


Fig. 5. [O_2] traces recorded during the oxidation of 10 μM 6-OHDA in the presence of 1 mM $AscH^-$. 1–6-OHDA alone; 2–6-OHDA added to $AscH^-$; 3 – $AscH^-$ alone; 4 – $AscH^-$ added to preoxidized 6-OHDA.

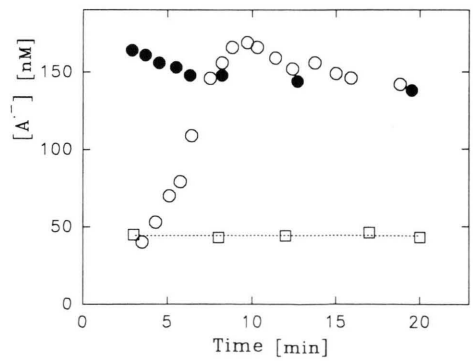


Fig. 6. Steady-state concentration of $Asc^{\bullet -}$ during the oxidation of 100 μM 6-OHDA in the presence of 1 mM $AscH^-$ at 21 °C. Symbols mean: ○ – 6-OHDA and $AscH^-$ are added simultaneously; ● – $AscH^-$ is added to preoxidized 6-OHDA; □ – $AscH^-$ alone.

compounds was reported repeatedly (O'Brien, 1991). For Q formed *via* CA oxidation, this was demonstrated in the current study.

The direct interaction of CA and 6-OHDA with molecular oxygen (the 'autoxidation' in a literal sense) is similar to other organic compounds (Miller *et al.*, 1990), spin-restricted, and the rate of reaction (3) at 37 °C is expected to be very low.

$$QH_2 + O_2 \longrightarrow Q^{\bullet -} + O_2^{\bullet -} + 2H^+. \qquad (3)$$

Thus so-called 'autoxidation' is a catalytic process. The catalytic action of Q on the oxidation of CA and 6-OHDA may be ascribed to the reaction

$$Q + QH_2 \longrightarrow 2Q^{\bullet -} + 2H^+ \quad k_4 \qquad (4)$$

followed by the interaction of $Q^{\bullet -}$ with oxygen

$$Q^{\bullet -} + O_2 \longrightarrow Q + O_2^{\bullet -} \quad k_5 \qquad (5)$$

As for CA oxidation, there is a kinetic challenge connected to the fact that o-$Q^{\bullet -}$ formed from CA display only moderate reactivity with oxygen ($k_5 < 1\times10^5$ M^{-1} s^{-1} (Kalyanaraman *et al.*, 1988)), in contrast to p-$Q^{\bullet -}$ (O'Brien, 1991; Kalyanaraman *et al.*, 1988). For this reason Kalyanaraman *et al.*, 1988 have suggested that the contribution of reaction (5) to CA oxidation is negligible and $Q^{\bullet -}$ decays mainly *via* the disproportionation

$$Q^{\bullet -} + Q^{\bullet -} + 2H^+ \longrightarrow Q + QH_2 \quad k_6 \qquad (6)$$

Contrary to the above opinion, the following rough estimation shows that under conditions of rather slow oxidation, reaction (5) predominates even when k_5 is as little as 1×10^5 $M^{-1}s^{-1}$. Assuming that the rate of $Q^{\bullet -}$ generation is equal to R_{OX} and a steady-state concentration of $Q^{\bullet -}$ is determined by its decay *via* reaction (6), a ratio of the rate of reaction (6) to that of (5) is given by the relation

$$R_6/R_5 = (R_{OX}\, k_6)^{0.5}/k_5\,[O_2].$$

At $R_{OX} = 1\times10^{-8}$ M s^{-1}, a typical value for the starting stage of CA oxidation (Table I), $k_6 = 1\times10^9$ $M^{-1}s^{-1}$ (O'Brien, 1991), $[O_2] = 2\times10^{-4}$ M and $k_5 = 1\times10^5$ $M^{-1}s^{-1}$, the calculated value of R_6/R_5 is about of 0.16. This is expected to be even lower as $Q^{\bullet -}$ decays not only *via* reaction (6) but also by (5). Computer kinetic simulations based on the expanded kinetic scheme confirm this conclusion (to be published elsewhere).

A contribution of Q into CA and 6-OHDA oxidation depends on two main factors: the rate constant for reaction (4) and a resistance of a primary o-Q formed by reaction (5) or (6) to intramolecular cyclization. A change of reduction potential in reaction (4), $\Delta E(4)$, is given by the combination of two one-electron potentials

$$\Delta E(4) = E(Q/Q^{\bullet -}) - E(Q^{\bullet -}/QH_2).$$

Unfortunately, neither $E(Q/Q^{\bullet -})$ nor $E(Q^{\bullet -}/QH_2)$ for species under study are available. Nevertheless, based on the values of half-wave two-electron reduction potential reported at pH 6.8 (Graham *et al.*, 1978) (+154 mV for 6-OHDA, +524 mV for DA, +544 mV for DOPA, +614 mV for NEP), one may suggest that $\Delta E(4)$ for 6-OHDA is clearly more positive than that of CA. Accordingly, with 6-OHDA reaction (4) is expected to occur much faster than with CA.

The character of the changes in R_{OX} with time basically depends on the resistance of primary Q (o-Q for CA (Graham, 1978; Hawley *et al.*, 1967) and both o- and p-Q for 6-OHDA (Köhle *at el.*, 1995)) to the intramolecular cyclization. Fast cyclization of a primary Q accompanied by its transformation in a non-quinoid compound may result in loss of a potential catalyst and thus prevents self-accelerating. In this case the effective autocatalysis is possible thanks to the accumulation of secondary Q (Q^I) through the involvement of Q^IH_2 into the oxidative transformation (see Scheme I). As the latter requires some time, the stage of an energetic self-acceleration is expected to be somewhat delayed. The following order of the aptitude of primary o-Q to the intramolecular cyclization was reported (Graham, 1978; Hawley *et al.*, 1967): 6-OHDA << DA < NEP ≈ DOPA < EP. Accordingly, 6-OHDA and DA, on the one, and EP, on the other hand, show the most energetic and most sluggish increase in R_{OX} , respectively, in the early stage of the oxidation; NEP and DOPA hold a middle position (Figs. 1 and 5).

Rather intricate kinetic regularities observed when CA is co-oxidized with $AscH^-$ may be explained by the simultaneous action of two factors: 1) inhibiting effect of $AscH^-$ on CA oxidation; 2) accelerating (catalytic) effect of quinoid products of CA oxidative transformation on $AscH^-$ oxidation. The first effect may be ascribed to the reversible reaction (1). In the case of CA, the equilib-

rium (1) is likely shifted to the right side. This follows both from the direct observations by the EPR method (Kalyanaraman *et al.*, 1984) and thermodynamic estimations. A change in reduction potential in direct reaction (1) with CA is likely positive. For unsubstituted catechol $E(Q^{\bullet -}/ QH_2)$ = + 530 mV at pH 7, and a single alkyl substituent may reduce this value by 50–100 mV (Wardman, 1989). As $E(Asc^{\bullet -}/ AscH^-)$ is as high as +282 mV (Wardman, 1989), $\Delta E(1)$ for reaction (1) given by

$$\Delta E(1)\ (mV) = E(Q^{\bullet -}/ QH_2) - 282$$

is expected between +200 and +150 mV favoring an equilibrium (1) shift to the right. Due to direct reaction (1) a steady-state concentration of $Q^{\bullet -}$ decreases and this results in lowering the rate of formation of Q in reaction (5) or/and (6) as well as oxygen uptake via reaction (5).

The question arises why, in contrast to CA, the oxidation of 6-OHDA is not inhibited by $AscH^-$. This is most likely connected with the dramatic difference in the thermodynamics of reaction (1) between 6-OHDA and CA. A value of $E(Q^{\bullet -}/ QH_2)$ for 6-hydroxy-DOPA (TOPA), a close structural analogue of 6-OHDA, was reported to be of +124 mV (Kano *et al.*, 1993)). This suggests that a value of $\Delta E(1)$ for reaction between $Q^{\bullet -}$ from 6-OHDA and $AscH^-$ is essentially negative and thus the equilibrium (1) may be shifted to the left, in contrast to that of CA.

A catalytic effect of oxidative products of CA and 6-OHDA on $AscH^-$ oxidation is most likely caused by the redox interaction between Q and $AscH^-$ (reaction (2)). The change in a reduction potential in reaction (2) may be given by the difference of two one-electron potentials

$$\Delta E(2) = E(Q/ Q^{\bullet -}) - E(Asc^{\bullet -}/ AscH^-)$$

or, having regard to $E(Asc^{\bullet -}/ AscH^-)$ is as much as +282 mV (Wardman, 1989),

$$\Delta E(2)\ (mV) = E(Q/ Q^{\bullet -}) - 282.$$

Values of $E(Q/ Q^{\bullet -})$ for primary Qs formed via the oxidation of CA and 6-OHDA are not available. A value of $E(Q/ Q^{\bullet -})$ was reported for adrenochrome (-253 mV (Wardman, 1989), a secondary Q formed from EP, and Q from TOPA (-232 mV (Kano *et al.*, 1993)), a close analogue of primary Q from 6-OHDA. For these Qs $\Delta E(2)$ is less than -500 mV and reaction (2) is expected to be rather slow. It is likely that $E(Q/ Q^{\bullet -})$, and thus $\Delta E(2)$ and k_2 for Qs of our study are not far from the reported values.

The following conclusions of biological significance may be reached from the above observations:

a) It is customary to assume that CA toxicity is caused by their pronounced aptitude to be oxidized by molecular oxygen. Considering the fact that a high content of CA in some tissues is combined with a normal physiological status of the body, the existence of some general way to prevent CA oxidation *in vivo* is anticipated. SOD is traditionally considered as an inhibitor of CA oxidation (Sachs and Jonsson, 1975; Gee and Davison, 1989) but SOD seems not to be potent enough to block CA oxidation at physiological pH. The results obtained in the present study allow to consider $AscH^-$ as the more potent and universal inhibitor of CA oxidation *in vivo*. The fact that tissues rich in CA contain an elevated amount of $AscH^-$ (Pileblad *et al.*, 1988) is an additional argument for this idea. An incidental reduction of [$AscH^-$] in nerve cells could result in a burst of CA oxidation leading to damage of these cells. Evidence supporting this scenario *in vivo* was recently reported (Fornstedt and Carlsson, 1991). The level of oxidative products of DA in guinea pigs which received an ascorbate-free diet was higher than that of those animals fed with a normal diet. A further significant increase of DA oxidation products was observed after the administration of a massive dose of $AscH^-$ to originally ascorbate-deficient animals.

b) In contrast to CA, 6-OHDA is known as an unconditionally neurotoxic agent. Generally, the pronounced toxicity of 6-OHDA is associated with its very high oxidizability (Bindoli *et al.*,1992; Sachs and Jonsson, 1978; Borg *et al.*, 1978). Based on this study we suggest that an additional reason for the high cytotoxicity of 6-OHDA is the fact that 6-OHDA oxidation is not inhibited by $AscH^-$.

c) Considering the molecular mechanism of neurotoxicity of 6-OHDA, particularly as a killing agent of neuroblastoma cells, special attention has been given to the fact that the main part of 6-OHDA administered is likely wasted due to the oxidation before penetrating into the target cells

(Bruchelt *et al.*, 1991; Gebhardt *et al.* 1993). We suggest that Q formed from 6-OHDA along with 6-OHDA itself are cytotoxic agents. Due to the high recycling effectiveness of the 6-OHDA – $AscH^-$ system one molecule of Q derived from 6-OHDA can induce the oxidation of a great number of $AscH^-$ molecules and provide the generation of active free radicals for a long period of time. Accordingly, $AscH^-$ can provide both the multiplication and prolongation of 6-OHDA cytotoxic action. This explains many essential features of 6-OHDA as a neurotoxin: the high effectiveness even at relatively low concentrations, a prolongation of toxic action as well as the enhancement of 6-OHDA neurotoxicity caused by adding $AscH^-$.

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