The oxidation of 6-hydroxydopamine in aqueous solution. Part 1. The formation of three metastable quinones at low pH †

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^{1}H and ^{13}C NMR investigations have been carried out in order to elucidate the products of oxidation of 6-hydroxydopamine [5-(2-aminoethyl)benzene-1,2,4-triol, protonated form H_3LH^+]. Stopped-flow, NMR kinetic experiments, and quantum mechanical calculations were employed as additional aids to the interpretation of the results. Evidence is provided that, at low pH, three quinones are produced that are in metastable equilibrium, namely the *p*- (*p*Q), *o*- (*o*Q), and triketo-quinones (tQ). Results obtained with sodium periodate and iron(III) are compared and discussed. At higher pHs (>2.5) the quinones reach rapid equilibrium because they start to deprotonate, all giving rise to the same species (Q^-), which is the only species detectable above a pH of about 6 and which is stable over a large pH range.

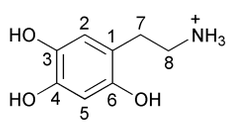
Introduction

6-Hydroxydopamine¶ [5-(2-aminoethyl)benzene-1,2,4-triol, protonated form H_3LH^+‖] is a catecholamine [3,4-dihydroxy-1-(2-aminoethyl)benzene] that, although closely related to the neurotransmitter dopamine, is neurotoxic. It is used in animal studies to investigate Parkinson's disease by causing significant damage to the dopaminergic neurons in a manner similar to that found in the disease.[1] The molecular mechanism that accounts for its neurotoxicity is unknown but it is thought to involve oxidative stress caused by the reduction and subsequent release of iron from ferritin.[2]

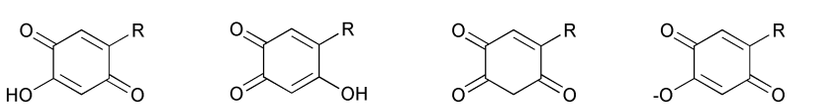
The present NMR study was carried out, however, in strongly acidic solutions, forming, as it does, part of a general investigation of the kinetics of the oxidation of 6-hydroxydopamine by aqueous ferric iron over a large pH range.

The oxidation of various catecholamines by aqueous iron(III) has been investigated by our group and their molecular mechanisms elucidated. Investigations with UV–Vis stopped-flow techniques have shown that dopamine,[3] dopa,[4] and noradrenaline[5] react *via* a semiquinone to form a stable *o*-quinone. 6-Hydroxydopamine, on the other hand, has been observed by us to form at least two products at different rates at a pH less than 4. It has been suggested before[6] that at low pH, 2,4,5-trihydroxybenzenes are oxidized to both the *o*- and *p*-quinones and that these are in equilibrium. The aim of this paper is to characterize the oxidation products of 6-hydroxydopamine at low pH (where they are amenable to study) and investigate their stability.

NMR was chosen as an unambiguous method of assigning the oxidation products. Although paramagnetic iron(III) is converted to diamagnetic iron(II) during the reaction, ^{1}H spectra are broadened by even the smallest quantities of Fe(III) ions. This does not allow easy assignment of signals and gives little or no information about any splitting. However, the necessary data on the nature of the oxidation products could be obtained by oxidizing with the diamagnetic periodate ion ($NaIO_4$). These



H_3LH^+
6-hydroxydopamine•H^+



pQ — *p*-quinone
oQ — *o*-quinone
tQ — triketo-quinone
Q^- — deprotonated-quinone

R = $CH_2CH_2NH_3^+$

† Time dependence data of the integrals in the ^{1}H NMR spectrum of 6-hydroxydopamine following oxidation by sodium periodate are available as supplementary data. For direct electronic access see http://www.rsc.org/suppdata/p2/b0/b007912j/

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¶ Note that 6-hydroxydopamine is not the correct name of this compound according to IUPAC guidelines. However, the greater part of the literature uses this name in order to clearly show its relationship to the parent catecholamine. We have therefore adopted this terminology.

‖ For convenience, –OH protons are written to the left, and the $-NH_3^+$ proton to the right of L.

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experiments with periodate could also be replicated using our standard UV–Vis stopped-flow techniques, thus enabling direct comparisons between the two methods to be drawn. Some simple ^{1}H NMR experiments with iron(III) were possible, however, and were undertaken in order to investigate the effect of another oxidant.

Experimental

NMR experiments were carried out using Bruker AC-250 and AMX-300 spectrometers (^{1}H resonance frequencies of 250 and 300 MHz respectively). The 6-hydroxydopamine used came from Sigma-Aldrich and from Fluka. $NaIO_4$, D_2O (99%), DSS, DCl (99%), NaOD (99%), and KCl were obtained from Aldrich. The DSS (sodium salt of 3-(trimethylsilyl)-propane-1-sulfonic acid) was used as an internal standard. The measurements of pD were made before and after each experiment *in situ* by means of a special "NMR" pH electrode from Hamilton.

Solutions that were 25–35 mM in 6-hydroxydopamine, and in sodium periodate, or iron(III) were made up in 2 M potassium chloride while purging with argon. Deuterated hydrochloric acid and sodium hydroxide were used to achieve the required pD. Distilled water was used as required.

The kinetics of the transformations of the products of oxidation of 6-hydroxydopamine were followed over 20 hours both by direct observations of the changes in spectra and in an automatic mode employing the AU-routines of Bruker spectrometers. Stopped-flow experiments were carried out on an SX-17MV stopped-flow spectrometer with photo-multiplier detector from Applied Photophysics. Solutions of 6-hydroxydopamine and sodium periodate of varying concentrations were prepared at low pH using hydrochloric acid and potassium chloride was added in order to keep the ionic strength constant at 0.1 M.** All solutions were de-oxygenated with argon and then transferred to the stopped-flow apparatus by means of Hamilton gas-tight syringes.

Quantum mechanical calculations were calculated using B3LYP (exchange and correlation potential) with the 6-311g-(d,p) basis set under Gaussian 98–DFT.

Results and discussion

The ^{1}H and ^{13}C NMR spectra of 6-hydroxydopamine in D_2O

Because of the known instability of 6-hydroxydopamine towards dioxygen, it was necessary to collect NMR spectra of the catecholamine itself. Thus the efficiency of our de-oxygenation techniques could be confirmed and we could be certain of unambiguously assigning features in the spectrum to oxidation products.

The main features of the ^{1}H spectrum obtained at a pD ≤ 1.00 can be summarized as follows: ^{1}H, 250 MHz, D_2O, pD = 0.72, δ values: 2.86 (t, 2H, J = 6.96 Hz), 3.22 (t, 2H, J = 6.96 Hz), 6.56 (s, 1H), 6.79 (s, 1H). (Note that although the solution was coloured, no evidence of oxidation products was observable in the NMR spectrum recorded over long periods of time.)

The ^{13}C NMR spectrum shows eight signals corresponding to the eight carbon atoms in 6-hydroxydopamine: ^{13}C, 62.9 MHz, D_2O, pD = 0.47, δ values: 29.9 (C7), 42.6 (C8), 107.1 (C5, "disappears" after 3 days), 117.7 (C1), 121.0 (C2), 140.0 (C3), 146.5 (C4), 150.4 (C6). The resonances were assigned by carrying out a 2D gs-HC-HMBC †† experiment.[7]

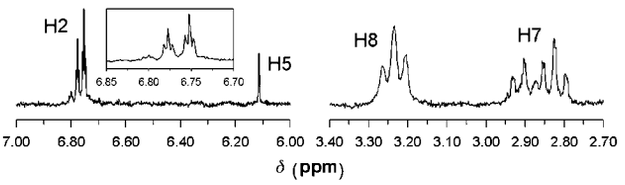
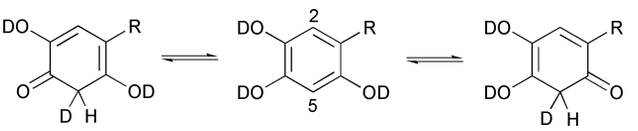


Fig. 1 The ^{1}H NMR spectrum of the initial products of oxidation of 6-hydroxydopamine by periodate in D_2O. (pD ≈ 0.72, both conc. = 30.2 mM.)

Over long periods of time the intensity of the H5 resonance decreased and after three days this signal completely disappeared while other signals (their positions and ratio of integrals) remained unchanged. These observations can be explained by deuteration *via* keto–enol tautomerization[8] (Scheme 1).



Scheme 1

This explanation is supported by experiments with solutions of 6-hydroxydopamine in non-deuterated water, the spectrum of which did not change: ^{1}H, 250 MHz, H_2O–10% D_2O, pH = 0.60, δ values: 2.85 (t, 2H, J = 6.96 Hz), 3.22 (non-resolved, 2H), 6.53 (s, 1H), 6.76 (s, 1H), 7.46 (very broad). Further evidence was provided by the ^{13}C NMR spectrum in D_2O, which showed an apparent disappearance of the signal corresponding to C5 (see above). A transformation of this signal into an unresolved triplet originating from C–D coupling was observed, however, after very many accumulations.

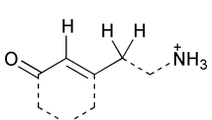
It is interesting that only position 5, and not 2, appears to be deuterated (although a feasible mechanism for both can be postulated). The obvious conclusion is that the two hydroxy groups *ortho* to position 5 make the proton there much more acidic than that at position 2.

Oxidation with sodium periodate (a two-electron oxidant)

When equivalent amounts of 6-hydroxydopamine and sodium periodate are mixed the solution turns a strong orange–brown, and the ^{1}H NMR spectrum recorded immediately after mixing (*i.e.* after about 1 minute) exhibits no signs of the signals due to the initial compound. This spectrum of the oxidation products, Fig. 1 (D_2O, pD = 0.72), contains at least two triplets at 2.82 (J = 7.2 Hz) and 2.90 ppm (J = 7.2 Hz) and these correspond to CH_2 protons at position 7 in at least two products and have a total integral of 2H. After about ten minutes, when the system has stabilized, these triplets are seen to be additionally split by 1.2 Hz, which arises from a long-range interaction with H2, typical for olefins.[9] This has been confirmed by experiments where these protons have been selectively decoupled.

A slightly broadened triplet at 3.24 ppm (t, 2H, J = 7.2 Hz) corresponds to the CH_2N at position 8 in both products. The fact that it appears as only one triplet indicates that there is no ring closure or condensation, caused by nucleophilic attack by the amine.

A singlet at 6.11 ppm (H5, products) vanishes in a short but measurable time of about 5 minutes due to deuteration. Triplets corresponding to H2 at 6.75, 6.77 and 6.80 ppm all have a splitting of J = 1.2 Hz because of their interaction with the CH_2 at 7 (as already stated above). The sum of their integrals is 1H and shows that three different structures are formed all containing the fragment shown below.



** A study of the kinetics has subsequently shown (see Part 3[23] in this series) that the ionic strength barely affects the rate. A comparison of the NMR, where a high ionic strength was needed in order to keep the pH constant, and stopped-flow experiments is therefore valid.

†† gs-HC-HMBC = gradient selected heteronuclear multiple bond correlation.

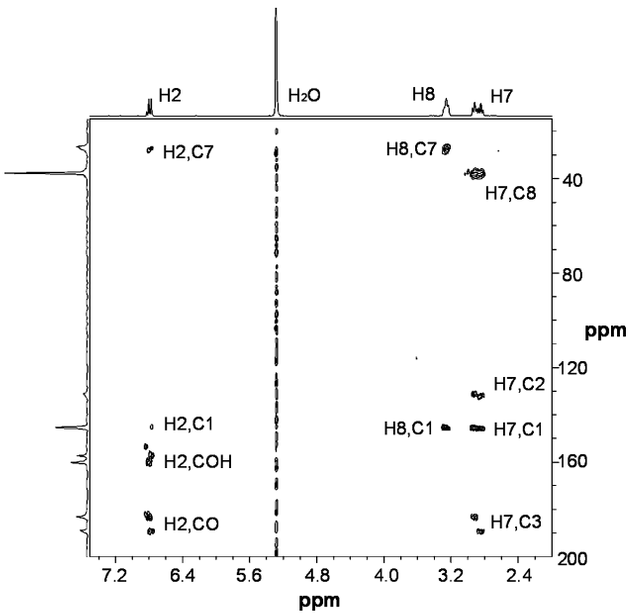
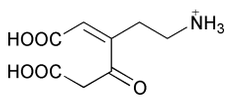


Fig. 2 The 2D gs-HC-HMBC spectrum of the first oxidation products of 6-hydroxydopamine. [6-Hydroxydopamine] = [$NaIO_4$] = 154.0 mM, pD = 0.37, 278 K. (Note that ^1J coupling has been suppressed and that the ^{13}C NMR spectrum to the left is a projection.)

Two of the species can be assigned to the *o*- (*o*Q) and *p*-quinones (*p*Q) but what could the third be? Ring cleavage could not be initially discounted because this is a common occurrence for *o*-quinones when such a strong oxidant is used.[10]



However, the carboxy groups of related compounds[11] all have a carbon chemical shift of 160–170 ppm, which is markedly different from the experimentally observed resonances at 180–207 ppm.

Neither is it possible that a ring-closed structure resulting from nucleophilic attack of the nitrogen atom at C2[3–5,12] is being produced because this would change the chemical shift of the aliphatic NCH_2 protons significantly and one would observe several such triplets.

Protonation at one or more of the oxygens, *e.g.* the protonated *o*-quinone,[3–5,12] is unlikely because at the low pH employed proton exchange is very fast and would average the signals corresponding to the protonated and non-protonated forms. One possibility, however, is protonation at one of the carbon atoms giving rise to a triketo form (tQ) because such proton transfers are known to be slow.[13]

Unfortunately, the ^{13}C NMR spectrum of the oxidation products in D_2O could not be obtained within a reasonable time on available spectrometers. However, a 2D gs-HC-HMBC experiment provided for sufficient sensitivity and easy assignment of the signals in the ^{13}C NMR spectra (see Fig. 2). It was carried out at 5 °C in order to slow the reaction sufficiently (the time required to carry out such an experiment was approximately $2\frac{1}{2}$ hours). Under these conditions, a broad-band decoupled spectrum of the oxidation products was also obtained from a solution in normal water. In this case, sensitivity was increased dramatically by an NOE due to the protons present [^{13}C, 62.9 MHz, H_2O–10% D_2O, pH = 0.37, δ values: 26.8, 27.4 and 28.1 (C7), 38.0 (C8), 87.8 and 108.9 (C5), 131.1 and 131.7 (C2), 145.2 and 145.4 (C1), 157.1 and 160.2 (COH), 179.7, 183.1, 184.3 and 207.4 (CO)].

In the gs-HC-HMBC spectrum ^1J(H, C) connections are suppressed but ^2J(H, C) and ^3J(H, C) correlations are allowed.

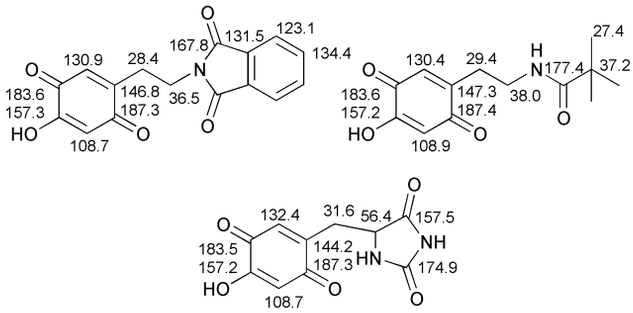


Fig. 3 The structures of some related *p*-quinones and their ^{13}C chemical shifts measured by Wang *et al.*[14] in DMSO.

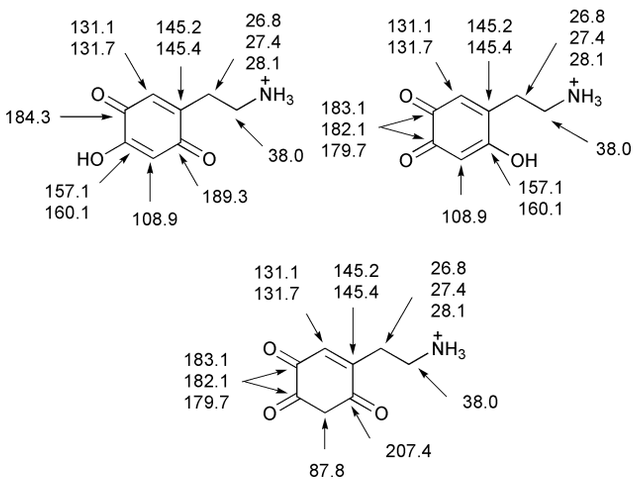


Fig. 4 The three quinones produced during the oxidation of 6-hydroxydopamine at low pH, and their ^{13}C chemical shifts measured in D_2O. Complete assignment was not possible and so carbon atoms have more than one value assigned to them.

Cross-peaks are therefore only observed for H8, C7 and H7, C8 in the aliphatic region. A weak cross-peak at (6.8, 28 ppm) corresponds to H2, C7; and at (2.8–2.9, 130–131 ppm) there are two for H7, C2 in different products. The latter resonances cannot arise from H7, C6 because that must be connected to a hydroxy or a carbonyl, which would be found at a higher ^{13}C chemical shift. Indeed the value of 130 ppm for C2 is comparable with similar *o*- and *p*-quinones measured by Wang *et al.*[14] (see Fig. 3), Svensson,[15] and Mure and Klinman.[16,17,18]

At 145 ppm there are cross-peaks for the H2, H7 and H8 protons. H8 is only able to interact with C1 and the chemical shift is comparable with literature values. Cross-peaks with H2 at approximately 160 ppm are characteristic of hydroxy groups.[19] As shown in Fig. 4, all three species have a carbonyl at position 3, but hydroxy groups can be found at positions C4 and C6. The fact that there is no cross-peak for H7, C6 is remarkable.

Chemical shifts of 183 and 187 ppm are reported for similar *p*-quinones.[14,16,17] A hydroxy group in the *ortho* and *para* positions is known to decrease ^{13}C chemical shifts in aromatic compounds, therefore the line at 183 ppm can be assigned to the carbonyl in position 3. The cross-peak at 187 ppm exists for the proton resonance at 6.75 ppm alone. Both the *o*- and *p*-quinones must show H2, C4(6) cross-peaks in the carbonyl region. One of these resonances is therefore hidden under the signal at 6.77, 183 which is indeed stronger than that at 6.75, 187 ppm. The presence of an alkyl group at C1 selectively lowers the chemical shift of the C4 carbon. It can therefore be concluded that the peak overlapping with H2, C3 is that arising from H2, C4 coupling. This allows us to assign the triplet in the proton spectrum at 6.75 ppm to the *p*-quinone and that at 6.77 ppm to the *o*-quinone. Smaller triplets in the proton spectrum (as will become obvious later) belong to the triketo form. The

Table 1 Total energies, HOMO and LUMO energies and the energy gap of the protonated and deprotonated forms of *p*Q and *o*Q. (Protonation occurs on the nitrogen atom)

	E/kJ mol^{-1}	E_{HOMO}/kJ mol^{-1}	E_{LUMO}/kJ mol^{-1}	$\Delta_{HOMO-LUMO}$/kJ mol^{-1}
*o*Q	−1552095	−924	−621	303
*p*Q	−1552218	−1044	−711	334
*o*Q(−H$^+$)	−1551148	−645	−330	315
*p*Q(−H$^+$)	−1551213	−651	−345	306

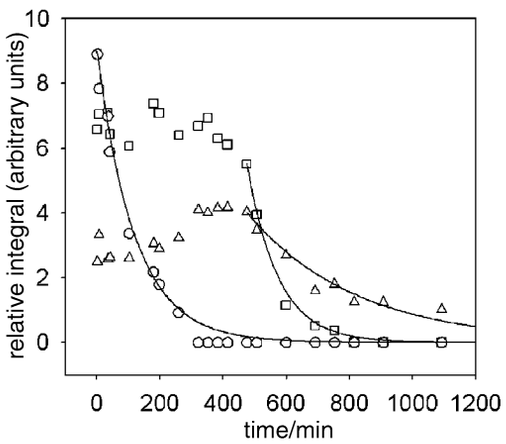


Fig. 5 The conversion of the quinones over time. (^{1}H Integrals from the supplementary data, taken relative to the DSS internal standard, which was given an arbitrary integral of 10.) ○ = triplet at 6.75 ppm (*p*Q); □ = triplet at 6.77 ppm (*o*Q); △ = triplet at 6.80 ppm (tQ).

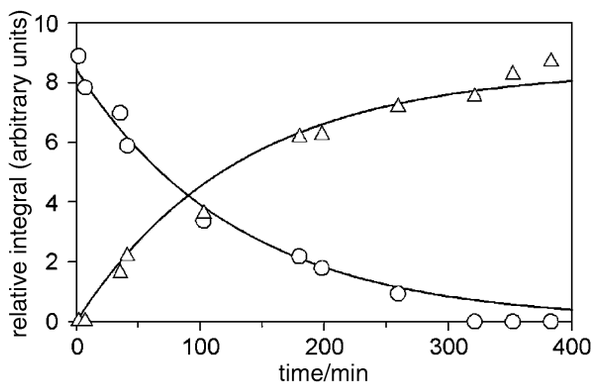


Fig. 6 The disappearance of the ^{1}H triplet at 6.75 ppm (○) arising from *p*Q and the concomitant appearance of a triplet at 3.40 ppm (△) due to ring closure. Both are fitted to a rate constant of 1.28×10^{-4} s^{-1}. (Integrals were taken relative to the DSS internal standard, which was given an arbitrary integral of 10.)

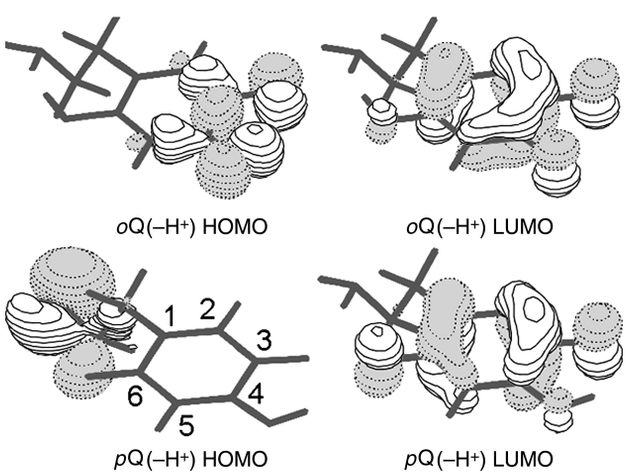


Fig. 7 The HOMOs and LUMOs of the deprotonated *o*- and *p*-quinones shown as 3D isosurfaces (+ve lobes are in grey). Calculated using B3LYP with the 6-311g(d,p) basis set, Gaussian 98–DFT. Note that the *p*-quinone HOMO is localized on the nitrogen.

weak cross-peaks at *ca.* 182 ppm correspond, therefore, to the carbonyl in position 3 in this structure.

The only carbons that are not assigned are C5, and C6 in the triketo-quinone. It is not surprising that the C5 carbon is not observed because this position is quickly deuterated and more than 3 bonds away from any protons. In fact, a $^{13}C\{^1H\}$ NMR spectrum in H_2O shows a strong resonance at 110 ppm not present in the 2D spectrum. Due to its large chemical shift and considerable NOE this signal can be ascribed to C5 in the *o*- and *p*-quinones. The triketo structure has two protons attached at C5 and thus a strong NOE is expected at lower chemical shift. However, only a weak signal is observed at 88 ppm indicating that the triketo form is present in very small quantities. The signal of the carbonyl at C6 and/or C4 is seen in this spectrum at 207 ppm thus reflecting the aliphatic and ketone characteristics of this quinone. Structures of the three oxidation products and assignments of NMR signals are given in Fig. 4.

Further transformations of the oxidation products

Over a longer time scale, there is no doubt that the iodate ions (IO_3^-), produced by the fast initial oxidation, are able to further oxidize the products. This was confirmed by following the time dependence of the ^{1}H NMR spectra (see supplementary data). The integral of the singlet corresponding to DSS was assigned an arbitrary value of 10. All other resonances were then integrated relative to this. Only the integrals of the triplets corresponding to H2 are plotted in Fig. 5 because they are the only signals that refer unambiguously to the quinones.

By the end of the time scale shown in Fig. 5 a substantial amount of melanin (a polymeric oxidation product) is formed. Nevertheless more than half of the initial amount of organic substance still remains in solution and yields a large number of triplet signals in the "aromatic" part of the ^{1}H NMR spectrum.

One sees immediately that, after a long time, all the initial quinones, bar one, disappear. The quinone present in the smallest amount in the initial mixture survives. According to the assignment given above this is the triketo form, which seems therefore to have a higher stability towards further oxidation and/or condensation (and this adds further weight to the observation that these quinones cannot be simple tautomers in rapid equilibrium). The signal at 6.75 ppm has been assigned to the *p*-quinone and it disappears (Fig. 5) relatively quickly with an accurate first order rate constant of 1.28×10^{-4} s^{-1}. What is more, this can be shown to coincide with a new ^{1}H triplet at 3.4 ppm (NCH_2 protons) (see Fig. 6). In other words, this quinone undergoes ring closure. This reaction involves nucleophilic attack at C6 by the nitrogen and its probability depends on the energy difference of the HOMO and LUMO and the "populations" on these atoms. Quantum mechanical calculations (Gaussian 98–DFT) performed for the *o*- and *p*-quinones (Table 1) show that the *p*-quinone in the uncharged form has a smaller energy gap, suggesting greater overlap in the transition state. Furthermore, by inspection of the HOMOs as 3D isosurfaces it is clear that only the *p*-quinone, deprotonated at the nitrogen, has significant electron density on the nitrogen (see Fig. 7). (Interestingly, even the deprotonated *o*-quinone

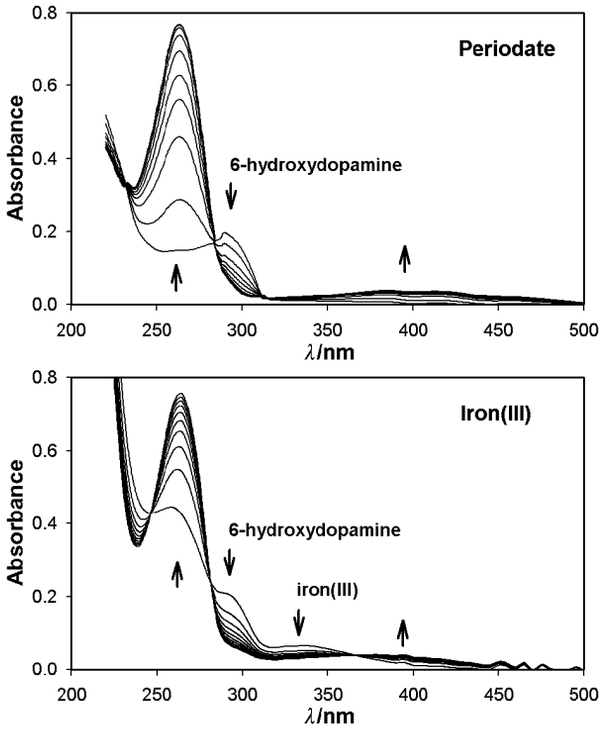


Fig. 8 The time dependent spectra of the oxidation of 6-hydroxydopamine by periodate (0–100 s, log time scale) and iron(III) (0–900 s, log time scale). pH = 1.00, [6-hydroxydopamine] = [periodate] = 0.050 mM, [iron(III)] = 0.10 mM. (Note that the bands at 390 and 265 nm increase at different rates for both oxidants.)

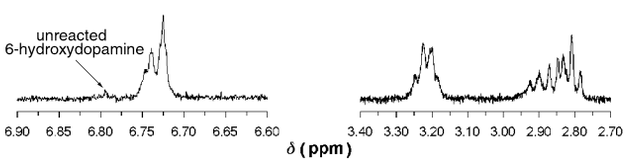


Fig. 9 The ^{1}H NMR spectrum of the oxidation of 6-hydroxydopamine by iron(III). pD = 0.65, [6-hydroxydopamine] = 33.2 mM; [iron(III)] = 47.6 mM.

lacks electron density on the HOMO centred on the nitrogen.) In keeping with experiment, therefore, theory predicts that the unprotonated *p*-quinone undergoes ring closure. This also explains the higher reactivity of 6-hydroxydopamine at higher pH.

A comparison of a one-electron and a two-electron oxidant

The time dependent spectral changes during the oxidation of 6-hydroxydopamine by comparable stoichiometric amounts of sodium periodate and iron(III) are given in Fig. 8. At this low pH the changes are comparable but the experiments were over different time scales (approx. 100 s for periodate and 900 s for iron(III)). (A full discussion of the significance of these absorptions is presented in Part 2 of this series.)[20]

The ^{1}H NMR spectrum of the products of oxidation by Fe(III), Fig. 9, shows a combination of three unresolved triplets in the aromatic region indicating a similarity in the products of oxidation by Fe(III) and periodate. On the other hand, the variation of chemical shifts of the H2 proton in these products is smaller than in the products of oxidation by periodate. This can be explained by interaction of the products with the Fe(II) present.

In the aliphatic region, there are at least two overlapping triplets due to NCH_2 protons and at least three triplets due to H7 protons. Additional signals can be ascribed to the excess of 6-hydroxydopamine (used in order to assure removal of the paramagnetic Fe(III) ions). However, the intensity of additional triplets in the aliphatic region is significantly larger than that expected from the intensity of the additional singlet in the aromatic region. A condensation reaction cannot, therefore, be ruled out.

Conclusions

It has been shown that the oxidation of 6-hydroxydopamine at low pH results in three quinones irrespective of whether a one-electron or two-electron oxidant is used. This fact is surprising but indicates that the transition states with both oxidants, and for each product (which must initially be semiquinones), are more "product-like" than "reactant-like". The ratio of the concentrations of the three semiquinones is therefore oxidant independent and arises from the way in which the unpaired electron and the protons are distributed over the molecular skeleton. This further implies that although periodate is an overall two-electron oxidant, the electrons are not transferred simultaneously. Indeed, it has been established that periodate is capable of acting *via* an I(VI) species in the oxidation of P(III) to P(V)[21] and the reduction of iodate by V(II) also proceeds by one-equivalent changes.[22] Finally, this does not rule out the probability that the semiquinone formed depends upon the point of attack and this would be further supported by the observation (see Part 3[23]) that the *o*- and *p*-quinones are formed at different rates (k_1 and k_2) (*i.e.* if these rate constants refer to the formation of the semiquinones as the rate-determining steps).

Employing 2D NMR it was possible to assign a number of signals in the ^{13}C NMR spectra of the reaction mixture containing these three quinones. Initially the *p*-quinone is produced in greatest amount, but it then disappears relatively quickly *via* a nucleophilic attack of the amine side chain. (This internal ring-closure reaction is a common feature of the oxidation products of the catecholamines.[3–5,12]) Similar transformations (which occur more slowly) are undergone by the *o*-quinone and the triketo structure (the latter can be considered to be a keto-isomer of either of the other two quinones.

A study of the kinetics (see Part 3[23]), has shown that the quinones are produced at different rates over similar time scales. This excludes the possibility that one quinone is produced which tautomerizes rapidly into the other two, and furthermore, the fact that the ratio of the quinones over much longer time scales is not constant (see Fig. 5) implies that the three quinones are metastable species.

Energetically (shown by DFT calculations) the *para*- and triketo forms are very close to each other (−1552218 and −1552153 kJ mol^{-1} respectively) whereas the *o*-quinone is significantly (55 kJ mol^{-1}) less stable. The attainment of equilibrium between these species is remarkably slow, indicating large barriers to protonation and structural rearrangements. At higher pH, the *o*- and *p*-quinones reach equilibrium rapidly *via* the common deprotonated quinone (Q^-) (see Part 2[20]).

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