Electron Spin Resonance Study of Semiquinones Formed during the Autoxidation of 6-Hydroxy-dopamine (2,4,5-Trihydroxy-phenyl-ethylamine)

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Z. Naturforsch. **50c,** 715–720 (1995); received July 5/July 27, 1995

EPR Spectroscopy, 6-Hydroxy-dopamine, Indoloquinones, Semiquinones

6-Hydroxy-dopamine is easily oxidized in aqueous solutions within a pH range of 7–14. Depending on this parameter and the starting concentration of this catechol at least seven different paramagnetic species can be detected. Cyclization to two different indole derivatives of aminochrome type could be demonstrated and the mechanism of formation is discussed. The indoloquinones can easily be reduced to the corresponding semiquinone by an excess of 6-hydroxy-dopamine. All radicals observed react with ascorbic acid in a redox reaction, forming the corresponding catechol and the ascorbic acid semidione.

Introduction

The selective destruction of nerve terminals of cells of the sympathetic nervous system by 6-hydroxy-dopamine is well known (Heikkila *et al.*, 1975). For this reason this compound is a widely used tool in neuropharmacology, and was used clinically as a purging agent of neuroblastoma cells prior to autologous bone marrow transplantation (Kushner *et al.*, 1991). Mechanistic studies are in agreement with the fact that reactive oxygen species – produced by non-enzymatic oxidation of the catechol moiety – play a key role (Graham *et al.*, 1978; Floyd *et al.*, 1979). In the course of these reactions the starting compound undergoes several redox reactions involving different semiquinones. For this reason we investigated the reaction of 6-hydroxy-dopamine with air oxygen by EPR spectroscopy in aqueous solution at various pH values.

Experimental

2,4,5-Trihydroxy-phenyl-ethylamine-hydrobromide (6-hydroxy-dopamine-hydrobromide) was purchased from Sigma (Munich) and used without further purification. Appropriate buffer solutions (Titrisol: pH 7 phosphate, pH 8 borate-hydrochloric acid, pH 9, 10, 11 boric acid/ potassium chloride-sodium hydroxide, pH 12 phosphate-sodium hydroxide, pH 13 potassium chloride-sodium hydroxide), L(+)-ascorbic acid, deuterium oxide and d-sodium hydroxide were obtained from Merck (Darmstadt).

The samples for EPR measurements were freshly prepared using buffer solutions previously deoxygenated by bubbling with argon for at least 10 minutes. After the addition of 6-hydroxy-dopamine the pH value was checked and, if necessary, adjusted by addition of 1 molar potassium carbonate solution. Air oxygen traces present in the EPR cell served as the oxidizing agent. Exchangeable protons were replaced with deuterium by using 1.0 N NaOD in D_2O. The spectra were recorded on a Bruker ESP 300 spectrometer equipped with a TM $_{110}$ cavity. The spectra simulation was done using a home made computerprogram written in C. By variation of the data directly obtained from the EPR or

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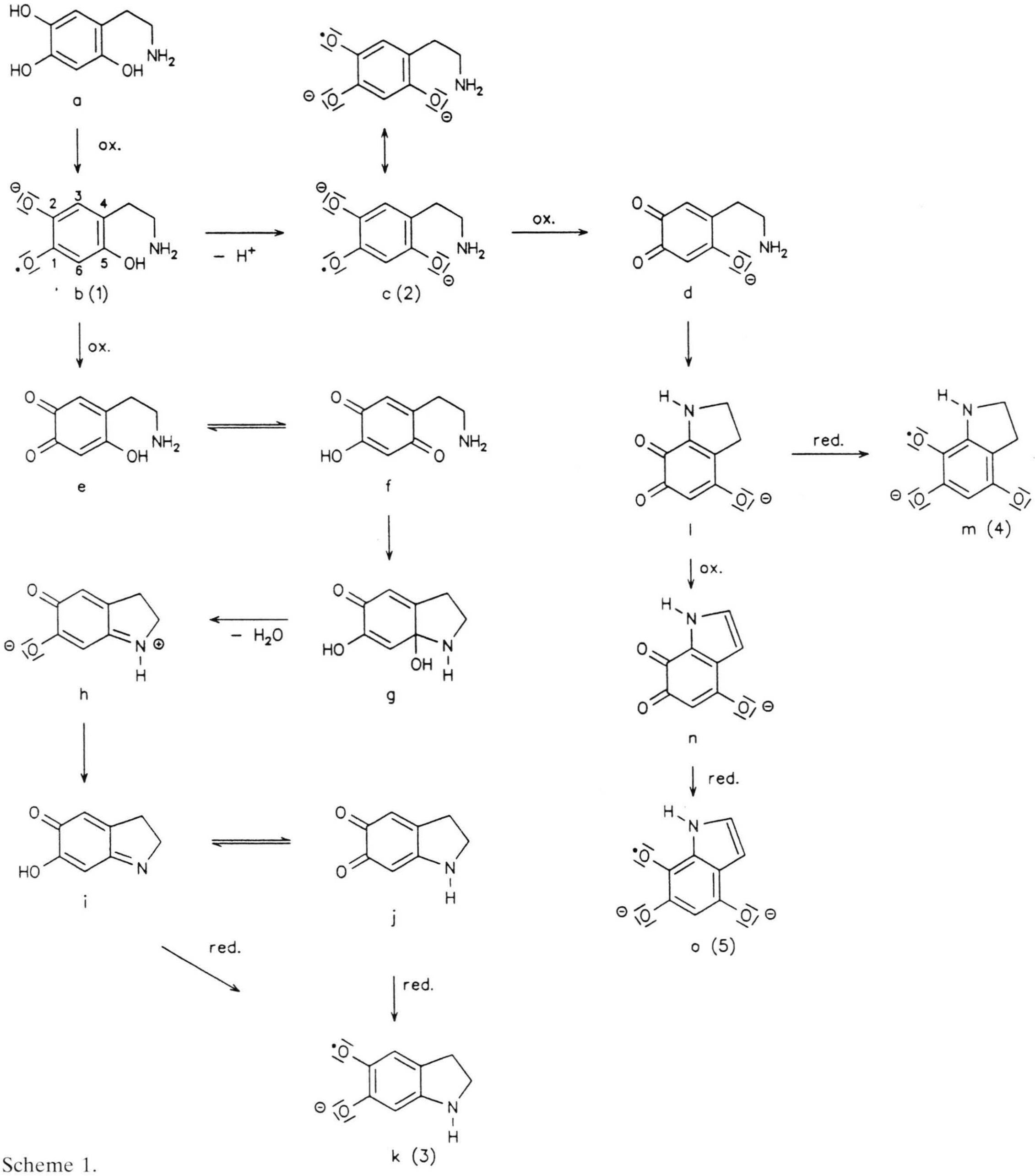
ENDOR spectra an optimal agreement with the experimental results was achieved.

Results

6-Hydroxy-dopamine is easily oxidized by air oxygen in aqueous buffer solution with a pH range between 7 and 14. At least seven paramagnetic species can be detected with the help of EPR spectroscopy. One of them (compound **c** in Scheme 1) shows the well known triplet of two doublets structure (not shown) which was attributed to the semiquinone dianion (Adams *et al.*, 1972). A very similar hyperfine structure (HFS)



Scheme 1.

was observed (Kalyanaraman *et al.*, 1985) using the spin stabilization technique. In pyridine solution (Stegmann *et al.*, 1985) the significantly smaller β-proton coupling constant indicates a different orientation of the side chain (Table I). Autoxidation in aqueous solution at pH values between 6 and 8 in absence of spin stabilizing ions results in a quite different EPR signal shown in Fig. 1. This hyperfine structure can be interpreted in terms of the interaction of the free electron with an additional proton (Table I).

One splitting of 0.42 G is missing in the spectrum taken in D_2O under the same experimental conditions (data not shown). For this reason we attributed this coupling to the hydroxy-proton in position 5 of the semiquinone.

Another paramagnetic species, the semiquinone **3**, could be observed during the investigation of the oxidation of the dopamine derivative over a period of several hours at pH 10. The extended hyperfine structure, with the overall width of ca. 35 G, indicates the interaction of the free electron with several different magnetic nuclei. This HFS is due to the interaction of the free electron with five types of protons and a nitrogen atom as shown in Table I. Three of them were not observed in the precursor **2** (see structure **c** in Scheme 1).

Prolonged slow oxidation of an 0.05 mol/l 6-hydroxy-dopamine solution with pH 13 leads first of all to the semiquinone **6**. The intensity diminishes within several days and a complex radical mixture is observed. Finally a new well reserved hyperfine structure is observed. This signal can be interpreted with the coupling constants given for radical **5** in Table I.

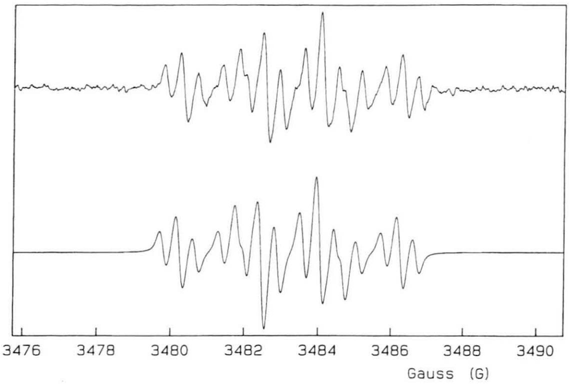


Fig. 1. EPR spectrum of the oxidation product **1** of 6-hydroxy-dopamine (0.05 mol/l) at pH 7. The spectrum obtained immediately after the addition of 6-hydroxy-dopamine to the phosphate buffer solution; above: experimental, below: simulation.

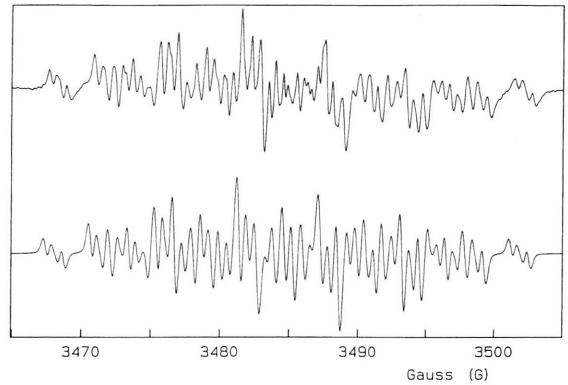


Fig. 2. EPR spectrum of the oxidation product **3** of 6-hydroxy-dopamine (0.05 mol/l) in buffer solution (pH 10) after prolonged oxidation; above: experimental, below: simulation.

Table I. Splitting constants in Gauß (G) and *g*-factors of various oxidation products of 6-hydroxy-dopamine.

No.[†]	Solvent	pH	a_{H_3}	$a_{H_4}^{(2)}$	a_{H_5}	a_{H_6}	a_N	$a_{NCH_2}^{(2)}$	a_{NH}	*g*	Ref.
1, b	H_2O	7	0.42	2.22	0.42	1.56	–	–	–	2.00425	a
2, c	H_2O	10	0.58	3.0	–	1.06	–	–	–	2.0043	a
3, k	H_2O	10	0.47	4.73	–	0.78	5.86	3.24	6.06	2.00348	a
	D_2O	10	0.47	4.65	–	0.78	5.78	3.24	0.94*	2.00348	a
4, m	H_2O	13	0.3	3.4	–	0.3	–	–	–	–	a
5, o	H_2O	13	0.22	2.70	–	1.00	0.24	–	0.6	2.0042	a
	D_2O	13	0.24	2.71	–	1.02	0.24	–	0.09*	2.00421	a
6, c	H_2O	13	0.57	3.36	–	0.85	–	–	–	2.0043	b,c
7	H_2O, Zn^{2+}	6	0.22	3.36	–	0.06	–	–	–	2.0040	a,c
8	Py, R_2Tl^+	–	+0.64	+2.55	–	−0.96	–	–	–	2.00355	d

[†] The letters correspond to the Scheme 1; * deuterium splitting; Py, pyridine; [a] this work; [b] Adams *et al.*, 1972; [c] Kalyanaraman *et al.*, 1995; [d] Stegmann *et al.*, 1985.

The structure and the formation of this paramagnetic species could be deduced from experiments in deuterated solution. Under these conditions first of all an additional radical characterized by a triplet of triplet hyperfine structure (radical **4** in Table I) is observed. In the presence of additional air oxygen the radical **4** undergoes another oxidation process indicated by EPR signal shown in Fig. 3. Autoxidation of 6-hydroxy-dopamine in D_2O buffer solution results in a significant change of the hyperfine structure as can be seen by comparison of Figs. 3 and 4. Both spectra can be easily simulated with the data given in Table 1.

All radicals observed react with ascorbic acid in a redox reaction. Addition of this reductant to the radical solutions, for example, addition of 0.15 mol/l ascorbic acid to an autoxidized 0.05 mol/l solution of 6-hydroxy-dopamine with pH 11 (EPR spectrum of radical **2**), leads to the reduction of **2** and the formation of the ascorbic acid radical, which means that the hyperfine structure shown in Figure 2 is replaced by that given in Fig. 5.

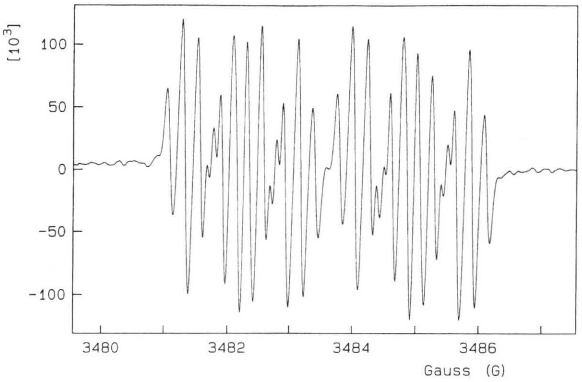


Fig. 3. EPR spectrum of the oxidation product **5** of 6-hydroxy-dopamine 0.05 mol/l in H_2O buffer solution (pH 13) after prolonged oxidation (ca. 14 days).

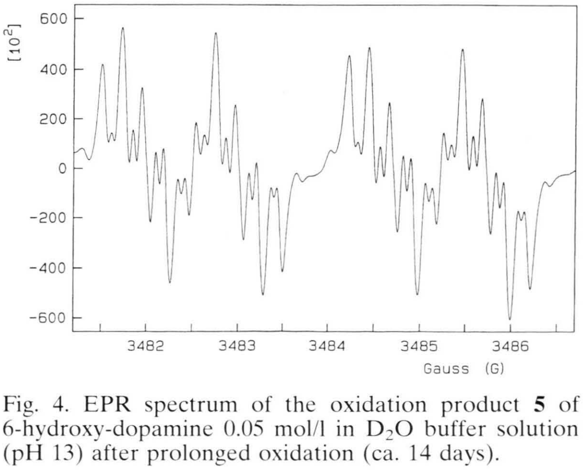


Fig. 4. EPR spectrum of the oxidation product **5** of 6-hydroxy-dopamine 0.05 mol/l in D_2O buffer solution (pH 13) after prolonged oxidation (ca. 14 days).

Discussion

Comparison of the coupling constants obtained for the radicals **6–8** with the values given for dopamine in the literature (Kalyanaraman *et al.*, 1985; Stegmann *et al.*, 1985) indicates an almost *o*-semiquinone spin density distribution in 6-hydroxy-dopamine semiquinone under these conditions (Table I). However, in a neutral medium radical **1** shows a remarkable variation of the coupling constants which can be interpreted in terms of an increasing contribution of resonance structures corresponding to para-semiquinone character. Spectra taken at intermediate pH values, for example pH 10 (**2** in Table I) confirm this interpretation.The relatively small variation of a_{H_3} could be due to a change in the sign of the coupling constants. However, TRIPLE resonance experiments in aqueous solution could not be done. A cyclic structure has to be assumed for radical **3** in all probability. Such indol derivatives could be detected by UV spectroscopy (Boschi *et al.*, 1977; Graham, 1978) but have not, to our knowledge, been isolated or characterized by more structure specific methods. The main oxidation product described in the literature is the acyclic quinone **6**. In contrast, EPR investigations of epinephrine, norepinephrine, dopa and analogous catechols after prolonged oxidation show unambiguously

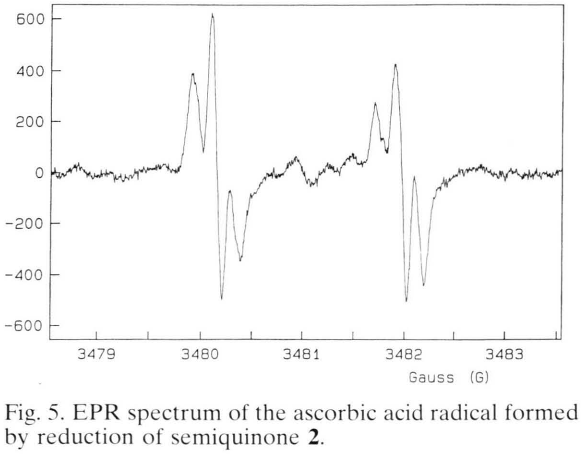


Fig. 5. EPR spectrum of the ascorbic acid radical formed by reduction of semiquinone **2**.

the formation of indolosemiquinones (Kalyanaraman *et al.*, 1985). In the case of 6-hydroxy-dopamine the analogous paramagnetic cyclic semiquinones are hitherto unknown. The structure of semiquinone **3** can easily be deduced from the splitting constants observed (Scheme 1, **k**). The two small coupling constants are typical for the aromatic protons in position 3 and 6 of *o*-benzosemiquinones (Klotz *et al.*, 1989). The interaction with the nitrogen atom is unequivocally seen in the spectrum. The proton with the largest splitting constant is exchangeable in D_2O. This spectrum (not shown) can be simulated with the parameters given in Table I. The remaining two pairs of protons $a_H^{(2)} = 3.24$ G and $a_H^{(2)} = 4.73$ G are therefore attributed to the two methylene groups of the heterocyclic ring (Table I and Scheme 1). In principle the assignment can be done in two different ways. Either the two equivalent protons can be attributed to the same methylene group or each carbon bears two protons with different hyperfine splittings. The first case requires – using the well known hyperconjugation equation (Scheffler and Stegmann, 1970) for systems with rigid geometry $a_{H_1} = B\,\varrho \cos^2 \theta_1 \,/\, a_{H_2} = B\,\varrho \cos^2 (\theta_1 + 120) = 1$ – dihedral angles for all four protons of 30 degrees. This leads to a coplanar geometry of the six- and the five-membered rings. If different coupling constants for the two protons adjacent to a distinct carbon atom are assumed then $a_{H_1}/a_{H_2} = 1.46$. This ratio can be associated with dihedral angles of approximately 21 and 39 degrees. In this case the five-membered ring would have a rigid twisted conformation. According to the large N and NH splittings there is a significant π-spin density at this heteroatom and therefore this nucleus adapts an almost π-sp^2 hybridization. That means the twisted five-membered ring consists of three sp^2 hybridized members and for this reason we favour the planar geometry.

From the EPR data obtained by the investigation of 4-methyl-5-dimethylamino-benzo-semiquinone-1,2 (Stegmann *et al.*, 1985) with freely rotating methyl groups, average $B\varrho$ values of 8.25 and 4.44 for the $C{-}CH_3$ and $N{-}CH_3$ groups respectively can easily be obtained. With these values hyperconjugation angles of 41 and 31 degrees were calculated using the assignment given in Table I. The agreement is sufficient if the different solvents, the presence of organothallium counterion and the ring structure of semiquinone **3** are taken into account.

The cyclization reaction as indicated by the intensity of the EPR signal of **3** depends on the pH value and the absolute concentration of the starting 6-hydroxy-dopamine. Solutions with a concentration of 10^{-3} mol/l and pH <9 show no EPR signal at all. In more alkaline buffers (pH 9, 10, 11) small concentrations of **3** can be detected. Raising the 6-hydroxy-dopamine concentration by a factor of 10 leads to a significant increase of the EPR signal intensity, which can be interpreted in terms of a superposition of the hyperfine structure of **2** and **3** obviously coexistent in a redox equilibrium. Further increase of the starting catecholamine concentration up to 1.4×10^{-2} mol/l at pH 8 leads to the formation of radical **1** followed by **3**, resulting in a mixture of both. The formation of **3** is favoured with decreasing proton concentration. However, at pH >11 no cyclic semiquinone **3** can be observed. In strong alkaline solutions (pH 13–14) only the spectrum of **6** with high intensity can be observed and no traces of the cyclic semiquinone **3** can be detected. Autoxidation of 6×10^{-2} mol/l solutions of 6-hydroxy-dopamine at pH 8 leads to the EPR signals of **1** in the first minutes, after which the signal is completely replaced by that of **3**. The rate of the cyclization reaction has a maximum at pH 10–11. These results enable us to deduce information concerning the mechanism of the indolosemiquinone formation (Scheme 1).

Monovalent oxidation of the catecholamine either by air or enzymatic oxidation leads to the corresponding semiquinone. The structure of this radical depends on the pH values of the solution. In a neutral or weak alkaline medium the protonated form **b** (**1**) is favoured whereas at pH >9 only the dianion **c** (**2**) can be detected. Further oxidation leads to the quinones **d** and **e**. The latter is tautomer to **f**. The anion **d** in consequence of its low electrophilicity does not have the capability for cyclization, via a structure analogous to **g**. For this reason no cyclic products of this type are observed at pH >11, but **f** can easily undergo an electrophilic attack in position 5 by the amino nitrogen leading to structure **g**. Protonation of the so formed OH groups takes place with a sufficient rate if pH <11. The resulting tautomeric quinones **i** and **j** respectively can easily be reduced to the semiquinone **3** which unambiguously has an ortho-semiquinone spin density distribution. The reductant for **j** obviously is the starting catechol **a** because enhanced radical intensities are observed with its increasing concentration. This interpretation is consistent with the observation of the radicals **4** and **5** (Table I) corresponding to structures **m** and **o** (Scheme 1). pH values >11 prevent a cyclization in position 5 due to the excess charge in the dianion, however, an addition in position 3 is feasible according to the EPR spectra. The radical **4** can be tentatively assigned to the first cyclization product. Reduction of the corresponding quinone yields the radical which is characterized by a triplet of the methylene protons in position 4. The expected small coupling constants of the other protons and the nitrogen cannot be detected due to poor resolution. Finally, in the course of the autoxidation a double bond is formed in the heterocyclic ring leading to the quinone **n**. Monovalent reduction by 6-hydroxy-dopamine results in the radical **5**. The corresponding EPR spectrum can be interpreted with the coupling constants given in Table I and the values would be consistent with the structure **o**.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) and the Volkswagen-Stiftung.

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