Effects of Redox Reagents on ESR Line Shape of Synthetic Melanins

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Melanins were synthesized from pyrogallol or β-(3,4-dihydroxyphenyl)alanine (dopa) and their electron spin resonance (ESR) spectra were measured. The pyrogallol melanin had four kinds of ESR signal; main and broad signals under anaerobic conditions, and inner and outer signals under aerobic conditions. The main, inner and outer signals disappeared by the reduction of the melanin by $NaBH_4$ or ascorbic acid but the broad signal did not. By the oxidation of the melanin by hexacyanoferrate(III), the intensity of the signals decreased, and the inner and outer signals were not observed under aerobic conditions. Hydrogen peroxide decreased these signals especially under aerobic conditions at high pH. Superoxide gave the signals similar to that under aerobic conditions, but decreased the signals of the hexacyanoferrate(III)-oxidized melanin. ESR signals of dopa melanin showed similar behavior to that of pyrogallol melanin against redox reagents. The chemical structure around radicals in melanins were proposed from these results.

The electron spin resonance (ESR) signals from a variety of melanins ranging from melanized human and animal tissues to synthetic melanins have been reported by many investigators.[1-11] These signals are due to the presence of an intrinsic radical in melanins and their signal strength has been known to be enhanced by UV and visible light.[2,4,5,11-16] Moreover, melanins have been found to consume molecular oxygen[17-20] and reduce it to superoxide anion radical[21,22] and to hydrogen peroxide.[23,24] These reactions are also enhanced by light.[17,22,24]

Melanins are soluble in basic aqueous solutions but not in acidic aqueous solutions nor in other inorganic and organic solvents. The ability of oxygen reduction by melanin and the radical concentration in melanins increase with increasing pH of the solutions.[9,12,17,23] The following reaction mechanism has been proposed for oxygen reductions by melanins.[23,25]

$$O_2 + \text{Melanin} \longrightarrow O_2^- + \text{Melanin radical (A)} \quad (1)$$

$$O_2^- + \text{Melanin} + 2H^+ \longrightarrow H_2O_2 + \text{Melanin radical (B)} \quad (2)$$

Because melanins contain high concentrations of reducing residues (hydroquinone moiety), they can form many radicals through one-electron oxidation.

It has been believed that the radical concentration in melanins is independent of oxygen concentrations in solutions.[9] However, Cope et al. have found that the oxygen concentration in the solution influences the radical concentration in melanins[12] and Chedekel et al. have reported that the ESR signal of a solution of pheomelanin from chicken feathers is very weak in the absence of oxygen but is strong in the presence of oxygen at pH 13.[26] This discrepancy should be clarified for the research of melanin chemistry.

There have been some studies on the effects of redox reagents on ESR signal intensity in melanins.[2,27,28] However, there have been no systematical and quantitative measurements of the effects of redox reagents on the ESR signal of melanins.

Although there have been many studies on melanins, the chemical structure of melanins has not been proved because of their complexity and of the insolubility of melanins in solvents.

In this article, we report the effects of O_2, O_2^-, H_2O_2, $[Fe(CN)_6]^{3-}$, dehydroascorbic acid, $NaBH_4$, ascorbic acid, and dihydronicotinamide-dinucleotide (NADH) on ESR signals in melanins synthesized from pyrogallol and β-(3,4-dihydroxyphenyl)alanine (dopa).

Experimental

Dehydroascorbic acid and potassium superoxide were obtained from Aldrich Chem. Co. and all other chemicals used were purchased from Wako Pure Chem. Industries, Ltd. and used without further purification.

Synthetic (untreated) melanins were prepared by autoxidation of pyrogallol and DL-dopa in deionized water of which pH was adjusted to 10 by the addition of concentrated aqueous ammonia.[9] Air was bubbled through the stirred solution for 3 d. After removing nonpolymerized compounds by precipitating melanins with 0.01 mol dm^{-3} hydrochloric acid, the solution was filtered off with a membrane filter (200 μm). The precipitates obtained were washed with deionized water and dried in vacuo over P_2O_5 for 72 h at room temperature to become a constant weight. The melanins so as to dissolve in buffer solutions were used as anhydrous powder to avoid preceding reactions with oxygen on storage.

Hexacyanoferrate(III)-oxidized melanins were prepared by mixing 500 mg of the synthetic melanins and 3.0 g of $K_3[Fe(CN)_6]$ in 500 cm^3 of 0.1 mol dm^{-3} phosphate-citrate buffer (pH 6.8) for 60 h at room temperature.[17] The solutions were acidified with concentrated hydrochloric acid to pH 2. After removing $[Fe(CN)_6]^{3-}$, the solution was filtered off and the precipitates were dried in vacuo as described above.

The buffer solution (pH 7.5—12.5) used was borate–citrate–phosphate buffer.[29] The pHs given in this article are final pH values after malanins were dissolved.

Untreated melanin or hexacyanoferrate(III)-oxidized melanin powder was dissolved in a buffer solution vigorously bubbled with argon in a sealed AtmosBag (Iuchi Co.), then the solution was introduced into a quartz flat cell (a JEOL LC-12)

to measure ESR. The residue of the argon saturated solution was vigorously bubbled with air for 10 min to examine the effects of molecular oxygen on the ESR spectra. ESR was measured in 2 min after the cessation of the bubbling.

To examine the effects of redox reagents on the ESR signals, untreated or hexacyanoferrate(III)-oxidized melanins together with redox reagents were dissolved in a buffer solution vigorously bubbled with argon. After 15 min ESR was measured. The residue of the solution was vigorously bubbled with air for 10 min, then ESR was measured in 2 min.

The reaction of superoxide with untreated melanins or hexacyanoferrate(III)-oxidized melanins was followed using a flow cell under argon atmospheres.[25] Buffer solutions of KO_2 (pH 13) and of melanins (pH 12.5) were mixed with a micro feeder Model JP-V (Furue Science Co.). The flow rate was fixed to 10.0 cm^3 min^{-1}.

ESR spectra were measured using a JEOL JES-FE1XG spectrometer operating at 9.5 GHz with 100 kHz field modulation. Spectra were obtained with a microwave power of 1 mW and a modulation amplitude of 0.10 mT. The ESR peak height in figures is expressed as an unit of cm/concentration of melanin (mg cm^{-3}) with a spectral gain of 2000. ESR spectra in figures are depicted with a spectral gain of 2000 unless particularly noticed. Mn(II) diluted with MgO and diphenylpicrylhydrazyl (DPPH) were used as a g marker. The ESR was measured at ambient temperature (15—20 °C) and nitrogen gas was flowing vigorously in a cavity to avoid the rise of temperature during measurement.

Results

Untreated Melanin. ESR signal of pyrogallol melanins dissolved in the solution saturated with argon showed a single line, which is named main signal hereinafter, with its width (peak-to-peak width of first-derivative signal) of 0.13 mT and its g value of 2.0037 accompanying a weak signal at a magnetic field lower than that of the main signal at pH 11.9 (Fig. 1A). The main signal was slightly unsymmetrical due to the presence of the above low field component at a high magnetic field where the main signal appeared. At a low pH (<9.8), the ESR signal consisted of a single broad (0.30 mT) line with g value of 2.0030 (Fig. 1B). This result suggests that the ESR signal of pyrogallol melanin consists of two lines; a main signal with its width of 0.13 mT and a broad one with its width of 0.30 mT, and that the main signal becomes weaker with decreasing pH. ESR signal of dopa melanin was similar to that of pyrogallol melanin with the exception of the linewidth of the main signal (0.30 mT) and of the broad signal (0.45 mT) (Fig. 1C and 1D).

Effect of O_2. When air was bubbled into the solution of pyrogallol melanin, two ESR signals explicitly appeared; inner one of which g value was 2.0037 and outer one of which g value was 2.0038 (Fig. 1A). The linewidth of the inner signal was about 0.083 mT when ESR spectra were measured with modulation width of 0.063 mT. The center of the outer signal localized at a slightly lower field (about 0.02 mT) than that of the inner one and its linewidth was about 0.50 mT, resulting in the spread of the signal on the both sides of the inner one. It is not clear at present whether the outer signal consists of two components or not. ESR spectra of dopa melanin under aerobic conditions showed two signals but the resolution was poor since the linewidth of the inner signal was broad (0.35 mT).

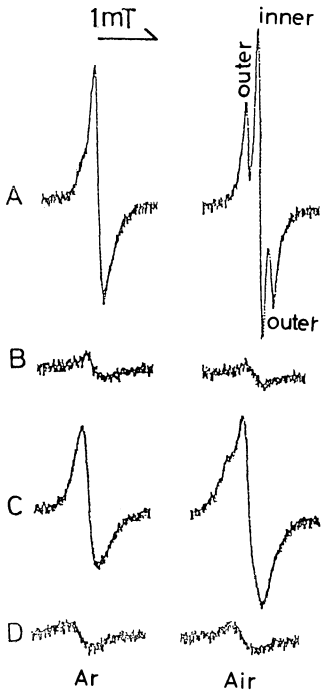

Fig. 1. ESR spectra of untreated pyrogallol (A,B) and dopa (C,D) melanins under anaerobic (Ar) or aerobic (Air) conditions. The concentration of melanins and pH values were A: 0.47 mg cm^{-3}, pH 11.9; B: 0.30 mg cm^{-3}, pH 9.4; C: 0.51 mg cm^{-3}, pH 12.5; D: 0.33 mg cm^{-3}, pH 8.1.

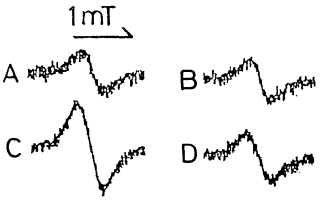

Fig. 2. ESR spectra of pyrogallol (A,B) and dopa (C,D) melanins reduced by $NaBH_4$ under anaerobic conditions. A: melanin 0.42 mg cm^{-3}, $NaBH_4$ 0.35 mg cm^{-3}, pH 10.6; B: melanin 0.38 mg cm^{-3}, $NaBH_4$ 0.38 mg cm^{-3}, pH 9.9; C: melanin 0.49 mg cm^{-3}, $NaBH_4$ 0.75 mg cm^{-3}, pH 11.8; D: melanin 0.37 mg cm^{-3}, $NaBH_4$ 0.71 mg cm^{-3}, pH 9.0.

Reaction with $NaBH_4$. When untreated melanins and $NaBH_4$ were dissolved in the solution saturated with argon, its ESR signal showed a weak and symmetrical single line in all the pH range examined (Fig. 2). Their linewidths were 0.30 mT for pyrogallol melanin and 0.48 mT for dopa melanin. These values were similar to those of the broad signals of pyogallol and dopa

Table 1. ESR Parameters of Synthetic Melanins[a)]

	Pyrogallol melanin		Dopa melanin	
	g Value	Linewidth / mT	*g* Value	Linewidth / mT
Main signal	2.0037	0.13	2.0036	0.30
Broad signal	2.0030	0.30	2.0031	0.48
Inner signal	2.0037	0.083	2.0036	0.30
Outer signal	2.0038	0.50	2.0037	0.51

a) The errors of *g* values are ±0.0005 and those of linewidths are ±0.005 mT.

melanins under anaerobic conditions (Fig. 1). This result suggests that radicals which contribute to the main signal are reduced by $NaBH_4$, whereas radicals which contribute to the broad signal are not reduced by $NaBH_4$. Table 1 shows ESR parameters of these four kinds of signal.

Hexacyanoferrate(III)-Oxidized Melanins. Figure 3 shows the ESR spectra of hexacyanoferrate(III)-oxidized melanins. The oxidized pyrogallol melanin showed an unsymmetrical signal with its linewidth of about 0.13 mT at a high pH and about 0.30 mT at a low pH similar to the linewidth of untreated pyrogallol melanin under anaerobic conditions, but the signal was weak compared with that of untreated pyrogallol melanin. The oxidized dopa melanin also showed an unsymmetrical signal with its width of about 0.32 mT at a high pH and about 0.45 mT at a low pH. Air bubbling caused the decrease of their signal strengths in all pH range as remaining line shapes unchanged (Fig. 3). The outer signals which were found in the ESR signals of untreated melanins under aerobic conditions did not appear by aeration. Sarna et al.[17] found that the oxidized dopa melanin consumes molecular oxygen at a high pH (>7.5). This and our data suggest that the

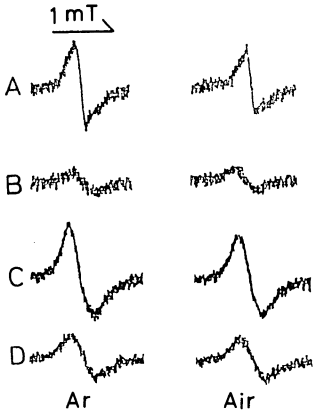

Fig. 3. ESR spectra of pyrogallol (A,B) and dopa (C,D) melanins oxidized by hexacyanoferrate(III) under anaerobic (Ar) and aerobic (Air) conditions. The concentration of oxidized melanins and pH values were A: 0.40 mg cm^{-3}, pH 12.3; B: 0.34 mg cm^{-3}, pH 8.6; C: 0.44 mg cm^{-3}, pH 11.9; D: 0.36 mg cm^{-3}, pH 9.7.

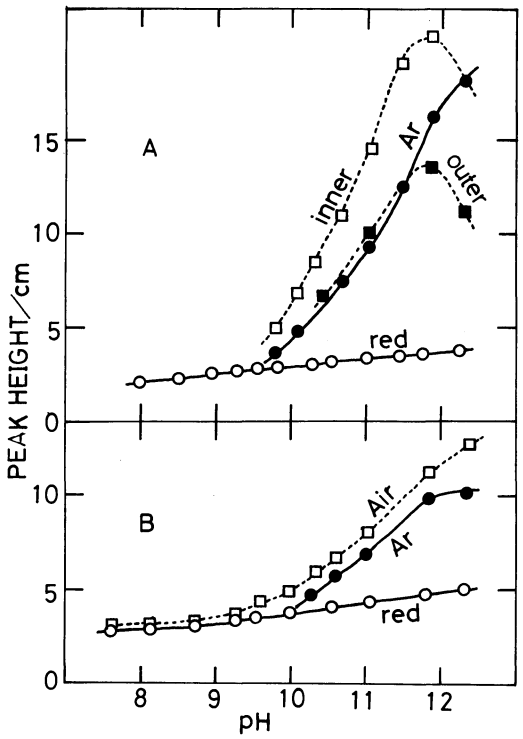

Fig. 4. The effect of pH on the peak height of untreated pyrogallol (A) and dopa (B) melanins under anaerobic (—●—) and aerobic (—□—, —■—) conditions, and that of $NaBH_4$-reduced melanins (—○—) under anaerobic conditions. The peak height of the outer signals of dopa melanin under aerobic conditions could not be estimated.

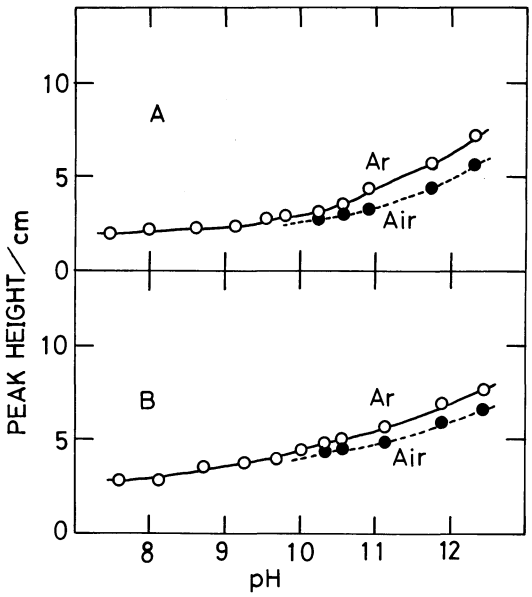

Fig. 5. The effect of pH on the peak height of pyrogallol (A) and dopa (B) melanins oxidized by hexacyanoferrate(III) under anaerobic (Ar) and aerobic (Air) conditions.

hexacyanoferrate(III)-oxidized melanin is further oxidized by molecular oxygen and that accordingly the concentration of a radical in the oxidized melanin decreases.

Figures 4 and 5 show effects of pH and oxygen on the ESR signal of untreated and $NaBH_4$-reduced melanins, and of hexacyanoferrate(III)-oxidized melanins. Since we could not integrate the ESR signals, their peak heights were compared. The strength of the broad signal of the $NaBH_4$-reduced melanins was not so dependent on pH of the solutions, whereas that of the main signal of untreated melanins was dependent on pH (Fig. 4). Air bubbling for 10 min of the solutions of untreated melanins enhanced their ESR peak height in all pH range. However, the height of pyrogallol melanin under aerobic conditions shrank at a high pH (>12). Since superoxide and hydrogen peroxide are formed (Eqs. 1 and 2) in the presence of oxygen and melanin, the effects of superoxide and hydrogen peroxide were studied to elucidate this shrinkage at a high pH.

Reaction with Superoxide. Figure 6 shows the effect of KO_2 on the signals of melanins at pH 12.8. The signals of untreated melanins were enhanced by KO_2. Pyrogallol melanin showed a signal similar to the signal of untreated pyrogallol melanin under aerobic conditions. The intensity of the signal of dopa melanin was also enhanced by KO_2. However, dopa melanin did not show any shoulder which was observed at a low field under aerobic conditions (Fig. 1). The decay rate of the free radical induced with KO_2 was not so fast compared with the data of Korytowski et al.[25] The signal strengths of hexacyanoferrate(III)-oxidized melanins were not enhanced by KO_2 but were weakened more rapidly than that of untreated melanins (Fig. 6B and 6D).

Reaction with H_2O_2. Hydrogen peroxide weakened the strengths of ESR signals of melanins keeping their line shapes similar to those before the addition of H_2O_2. Figure 7 shows the effect of the concentration of H_2O_2 on the peak height of melanins. The signal strength of melanins were weakened especially at a high pH under aerobic conditions. From this result, it is apparent that the decrease of the intensity of ESR signal of pyrogallol melanin under aerobic conditions at a high pH is due to H_2O_2 produced during aeration, since H_2O_2 formation from O_2 and melanins is rapid at higher pH values.[23]

Reaction with Ascorbate. When an excess amount of ascorbate (about 1.5 times of melanin by weight) was added to the untreated or the hexacyanoferrate(III)-oxidized melanin under anaerobic conditions, a new signal appeared at a low magnetic field and the intensity of the main signals of pyrogallol and dopa melanins (Fig. 1) decreased. However, the broad signals remained (Fig. 8A—8F). This new signal was assigned to

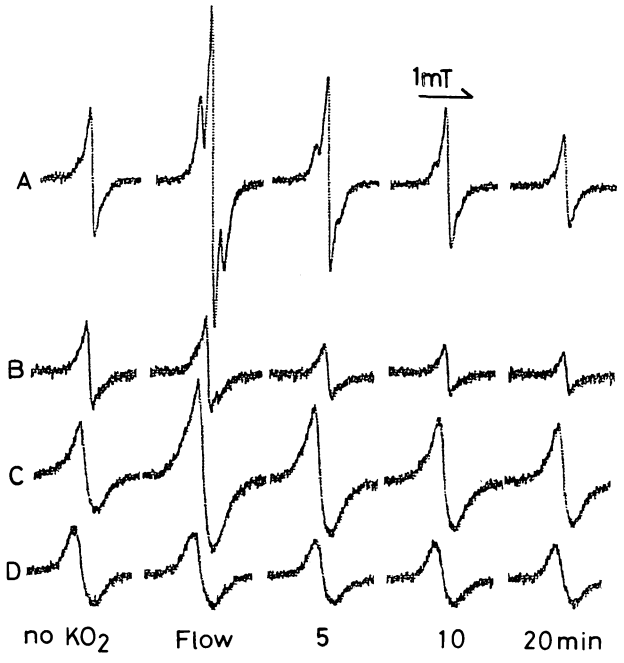

Fig. 6. ESR spectra of melanins during continuous flow (10 cm^3 min^{-1}) against 5.0 mmol dm^{-3} KO_2 in buffer solution. The final pH of the mixture was 12.8. The final concentration of melanins were A: untreated pyrogallol melanin 0.28 mg cm^{-3}; B: hexacyanoferrate(III)-oxidized pyrogallol melanin 0.26 mg cm^{-3}; C: untreated dopa melanin 0.25 mg cm^{-3}; D: hexacyanoferrate(III)-oxidized dopa melanin 0.27 mg cm^{-3}. The number in figure is time (min) after the flow was stopped.

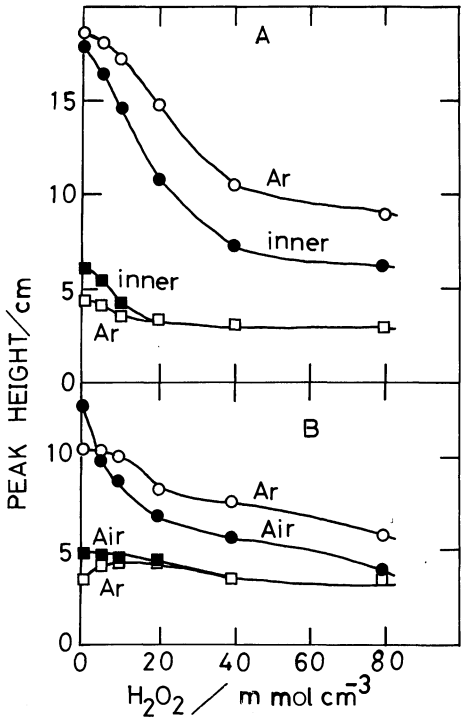

Fig. 7. The effect of the concentration of hydrogen peroxide on the peak height of pyrogallol (A) and dopa (B) melanins under anaerobic (Ar) and aerobic (inner or Air) conditions at pH 12.4 (—○—) and 9.9 (—□—). The peak height of the outer signal of pyrogallol melanin under aerobic conditions was not depicted.

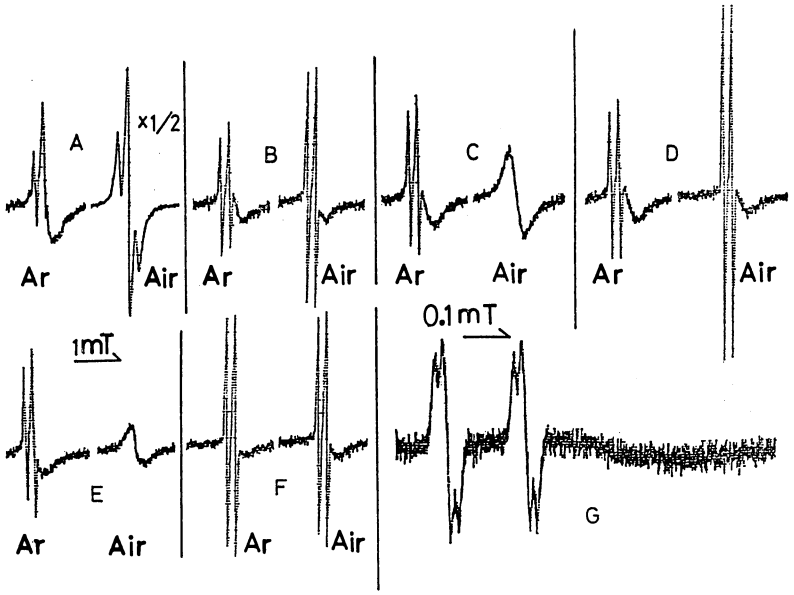

Fig. 8. ESR spectra of the mixtures of L-(+)-ascorbic acid sodium salt and pyrogallol (A,B), dopa (C,D) or oxidized pyrogallol (E,F) melanins under anaerobic (Ar) and aerobic (Air) conditions. The concentration of melanin, the ratio (ascorbic acid/melanin, (w/w)), and pH values were A: 0.57 mg cm^{-3}, 1.77, pH 11.6; B: 0.38 mg cm^{-3}, 1.87, pH 10.2; C: 0.49 mg cm^{-3}, 1.49, pH 11.9; D: 0.43 mg cm^{-3}, 1.65, pH 10.2; E: 0.29 mg cm^{-3}, 0.52, pH 10.0; F: 0.24 mg cm^{-3}, 4.42, pH 10.0, respectively, G: expanded spectrum of monodehydroascorbate signal at pH 9.2 measured with modulation width of 0.025 mT.

monodehydroascorbate radical[30-32] from measuring by the use of small modulation amplitude (Fig. 8G). This result suggests that ascorbate reduces a radical which contributes to the main signal but does not reduce a radical which contributes to the broad signal and that ascorbate reduces also the melanin oxidized by hexacyanoferrate(III). The pH effect on the peak height of the signal due to monodehydroascorbate radical (as an unit of cm/concentration of melanins (mg cm^{-3})) is shown in Fig. 9. The pH effect on the peak height of monodehydroascorbate was roughly correlated to that of the main signal of untreated melanins (Figs. 4 and 9). The steep down of the peak height at a high pH (>10.4) is perhaps due to that there is not enough ascorbate in the solutions to reduce the melanins or melanin radicals.

When the solutions of ascorbate and melanins were bubbled with air for 10 min, the signals of monodehydroascorbate disappeared and the strong signals similar to that of respective melanins under aerobic conditions appeared at a high pH (Fig. 8A, C, and E), whereas at a low pH the strong signals due to monodehydroascorbate remained and the intensity was enhanced (Fig. 8B, D, and F). This result suggests that in the course of air bubbling, there are occurences of the one-electron oxidation of melanins by molecular oxygen, one-electron reduction of the melanins by ascorbate and the oxidation of ascorbate to dehydroascorbate through

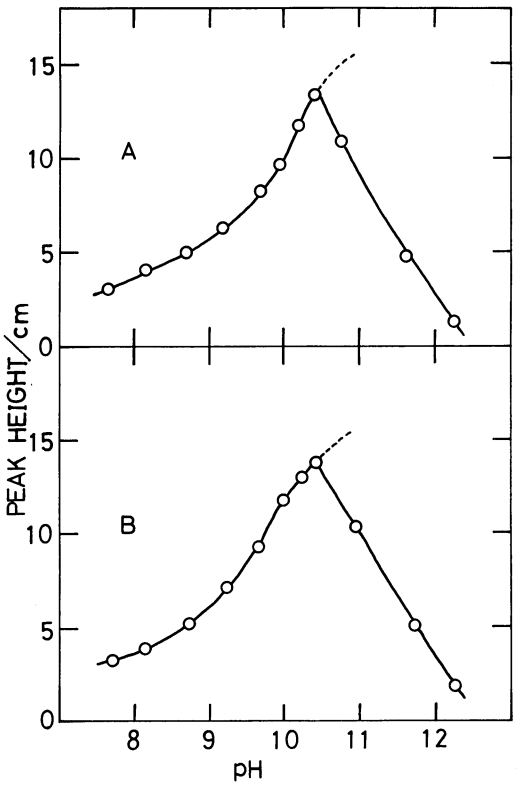

Fig. 9. The effect of pH on the peak height of low field monodehydroascorbate signal mixed with pyrogallol (A) and dopa (B) melanins in the argon saturated solution.

monodehydroascorbate.

To elucidate the reaction mechanism mentioned above, ESR spectra of the solution containing ascorbate and the pyrogallol melanin or the oxidized pyrogallol melanin were measured under anaerobic and aerobic conditions alternating the ratio of ascorbate/melanins (w/w) (Figs. 10 and 11). At pH 10.9, the peak height (cm/concentration of melanin (mg cm^{-3})) of monodehydroascorbate under anaerobic conditions approached at its maximum at the ratio of 3.0 (Fig. 10B). It is supposed that when air is bubbled in the solution, melanins are partially oxidized by O_2 and ascorbate is consumed to reduce the oxidized form of melanins. Accordingly at the ratio smaller than 2.0, the ESR signal of pyrogallol melanin remained at pH 10.9 because ascorbate dissolved in the solution was oxidized to dehydroascorbate. Whereas at the higher ratio (>3.0), the ESR signal of pyrogallol melanin disappeared and the strong signal (Asl) of monodehydroascorbate appeared (Fig. 10B).

The rates of the oxygen consumption,[17] H_2O_2 formation[23] and perhaps O_2^- formation[23] by melanin become smaller with decreasing pH. Therefore, the rate of oxidation of melanins by O_2 becomes smaller with decreasing pH. Accordingly at pH 9.8 (Fig. 10A), the disappearance of the ESR signal of pyrogallol melanin and appearance of the ESR signal of monodehydroascorbate under aerobic conditions were occured even at the lower ratio of ascorbate/melanin than at pH 10.9 (Fig. 10B), since the rate of the oxidation of ascorbate by melanins is slow at a low pH.

When a large amount of ascorbate (8 times of pyrogallol melanin by weight) was added to the pyrogallol melanin under aerobic conditions at pH 11.8, the inner and outer signals disappeared and the strong signal of monodehydroascorbate appeared, whereas the broad signal remained. When $NaBH_4$ instead of ascorbate was added under aerobic conditions, the inner and outer signals also disappeared and the broad signal remained (date not shown). This result suggests that a radical which contributes to the broad signal was different from a radical which contributes to the outer signal.

The peak height of monodehydroascorbate in the solution mixed with ascorbate and the oxidized pyrogallol melanin under anaerobic conditions (Fig. 11) was larger than that in the solution mixed with the

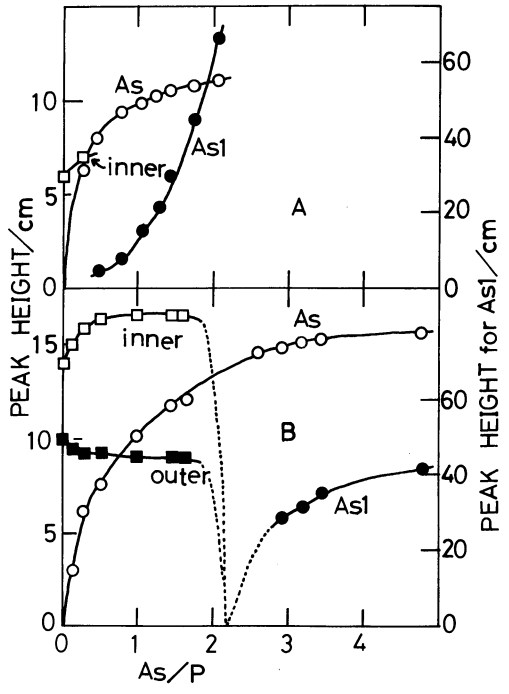

Fig. 10. The effect of the ratio (ascorbic acid/pyrogallol melanin, (w/w), As/P) on the peak height of monodehydroascorbate (—○—) under anaerobic conditions and of pyrogallol melanin (outer, inner) or of monodehydroascorbate (—●—) under aerobic conditions at pH 9.8 (A) and 10.9 (B). Asl in the figure shows the signal of monodehydroascorbate remained after air bubbling and As shows that under anaerobic conditions.

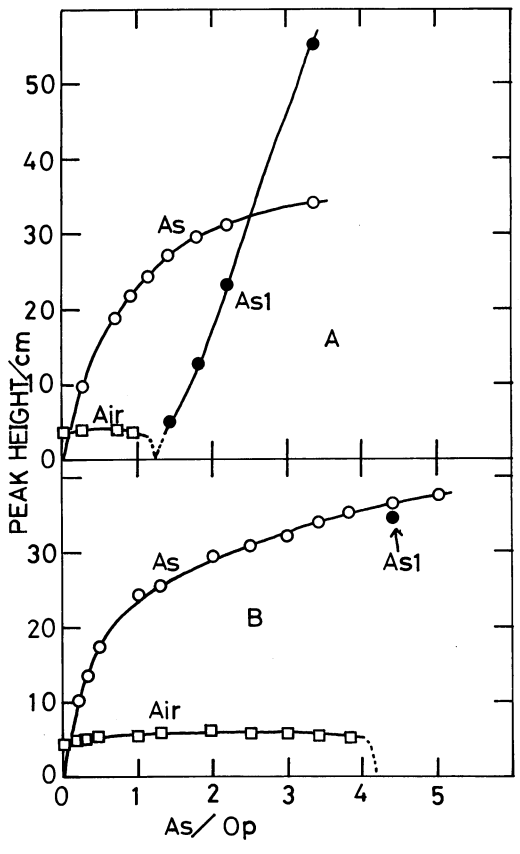

Fig. 11. The effect of the ratio (ascorbic acid/oxidized pyrogallol melanin, (w/w), As/Op) on the peak height of monodehydroascorbate (—○—) under anaerobic conditions and of pyrogallol melanin (Air) or of monodehydroascorbate (—●—) under aerobic conditions at pH 9.8 (A) and 10.9 (B). Asl in the figure shows the signal of monodehydroascorbate remained after air bubbling and As shows that under anaerobic conditions.

untreated pyrogallol melanin (Fig. 10). Under aerobic conditions, the signal of the oxidized pyrogallol melanin appeared even at larger ratios than in the case of the untreated pyrogallol melanin and the signal of monodehydroascorbate also appeared at large ratios. It was found that a large amount of ascorbate is required to reduce the oxidized pyrogallol melanin compared with the untreated pyrogallol melanin and that the oxidized pyrogallol melanin consumes molecular oxygen[17] resulting in the oxidation of ascorbate by the hexacyanoferrate(III)-oxidized melanin.

Reaction with NADH and Dehydroascorbate. NADH and dehydroascorbic acid did not change the strength and line shape of the ESR signals of untreated melanins in the pH range examined (pH 7.5—12.0). It has been found that NADH is oxidized by melanins at neutral pH values.[28,33,34] Van Woert found that the ESR signal of dopa melanin decreases its strength in the presence of NADH at pH 7.2.[28] Since we studied only ESR spectra in melanins at high pH, this discrepancy could not be elucidated.

Discussion

The ESR signal of dopa melanin gave a broad line and only an unsymmetrical line shape at X-band because of the overlap of some signals.[10] However, the behavior of the ESR spectra of dopa melanin was similar to that of pyrogallol melanin in its chemical modification. The ESR spectra of pyrogallol melanin gave a narrow line compared with dopa melanin and was separated into some signals even in X-band spectra. Therefore, we discuss the chemical nature of melanins using the results of pyrogallol melanin.

The ESR spectra of pyrogallol melanin gave at least four kinds of signal; the main signal and broad one under anaerobic conditions, and the outer signal and inner one under aerobic conditions. Grady and Borg[10] measured ESR spectra of dopa melanin at Q-band and explained that the main signal is due to the radical which dissociates proton at alkaline pH from a radical contributed to the broad signal, although it is not evident whether they measured ESR spectra under aerobic conditions or anaerobic conditions. However, it was found that the main signal of pyrogallol melanin is different from the broad signal in its fashion against chemical modification, i.e., the main signal disappeared in the reduction of melanin by $NaBH_4$ or ascorbate but the broad signal did not. The outer and inner signals disappeared by adding an excess amount of ascorbate. The data indicate that the radical which contributes to the broad signal is different from the radical which contributes to the outer signal, although their linewidths were similar to each other. It is not evident whether the radical which contributes to the main signal and the radical which contributes to the inner signal are the same one or not. The linewidth of the inner signal is smaller than that of the main signal. Oxygen molecule dissolved in the solution generally causes line broadening of ESR signals. Therefore, it is probable that these two signals are due to different radicals.[26]

The high concentration of quinone and hydroquinone units in melanins can form a quinhydrone type complex,[8,9,14] which partially dissociates into a radical in an aqueous solution through intra- or intermolecular interactions. Deprotonation from hydroquinone units in the complex at higher pH values stabilizes the quinhydrone type complex resulting in the increase of radical concentration. Temperature[3,8,14] and pH[9,12] dependences of ESR signal intensity in melanins have been reasonably interpreted using this concept.[14] We propose the chemical structure of radicals in melanins using this concept. It may be supposed that two different quinhydrone type complexes, complex A and complex B, contribute to the broad signal and the main signal, respectively, of pyrogallol melanin under anaerobic conditions.

The complex A is shielded internally by the bulky moiety resulting in the difficulty in the deprotonation at higher pH values and in oxidation and reduction by redox reagents at the complex A. Therefore, the intensity of the broad signal did not appreciably depend on pH of the solution (Fig. 4) and the broad signal did not disappear by modification with $[Fe(CN)_6]^{3-}$ (Fig. 3), $NaBH_4$ (Fig. 2), or ascorbic acid (Fig. 8).

The hydroquinone and quinone units loosely bind in the complex B, and are exposed to the solvent and to the redox reagents in the solution. The intensity of the main signal was eventually dependent on pH of the solution (Fig. 4) because of the deprotonation, and the main signal disappeared by $NaBH_4$ (Fig. 2) or ascorbate (Fig. 8) because of the reduction of the quinone unit of the complex B.

Free quinone and hydroquinone moieties which do not form a quinhydrone type complex make no radicals under anaerobic conditions.

Hexacyanoferrate(III) oxidizes the hydroquinone unit of the complex B and the free hydroquinone moiety. However, by the oxidation of the hydroquinone moieties, molecular structure changes to shield the hydroquinone unit of the complex B from the oxidation by hexacyanoferrate(III) because of delocalization of π-electron in benzene ring and of bulky size of hexacyanoferrate(III). Therefore, the oxidation of hydroquinone unit of the complex B by hexacyanoferrate(III) is inhibited half-way. Accordingly the hexacyanoferrate(III)-oxidized melanin showed the ESR signal which is similar to the signal of untreated melanin (Figs. 1 and 3) under anaerobic conditions but is weaker than the signal of untreated melanin (Figs. 4 and 5).

Ascorbic acid can reduce the quinone units of the complex B and the free quinone moiety. It was found that monodehydroascorbate decomposes to ascorbate and dehydroascorbate by comproportionation.[32] The comproportionation constant increases with increasing pH but has not been determined at high pH values (>6.4).[32] Therefore, the reaction mechanism between

melanins and ascorbate under anaerobic conditions needs further clarification taking into account of the comproportionation.

Molecular oxygen can oxidize the hydroquinone unit of the complex B and the free hydroquinone moiety, resulting in radical formation at the free hydroquinone moiety and in radical destruction at the complex B by aeration. The oxidized melanin in which the free hydroquinone moiety is already oxidized to the free quinone moiety by hexacyanoferrate(III) did not show the radical formation contributing to the inner or outer signal, but showed the radical destruction at the complex B by aeration (Figs. 3 and 5). However, it is not evident from our result whether the inner or outer signal should be assigned to the radical formed from one electron oxidation of the free hydroquinone moiety by aeration. Pyrogallol anion radical showed hyperfine structure, a_{5H}=0.54 mT and a_{4H}, a_{6H}=0.093 mT, in our buffer solution. When it is assumed that the outer signal is splitted into two components by hydrogen nucleus, its linewidth is estimated to be 0.07 mT. However, hyperfine structure of respective signal could not be observed even by the use of modulation width of 0.05 mT and the repeating of the dissolution and reprecipitation of pyrogallol melanin did not change the inner and outer line shape. It was concluded that the outer signal is characteristic of melanins. It is very probable that the outer signal is due to the radical in a pyrogallol moiety which binds to other moieties through 4- and 6-carbon atom in the benzene ring and is splitted into two components by 5-H hydrogen nucleus.

When air was bubbled in the solution of ascorbate and hexacyanoferrate(III)-oxidized pyrogallol melanin mixture at high pH, the signal similar to that of untreated pyrogallol melanin was not observed (Fig. 8A and 8E). It was found that hexacyanoferrate(III)-oxidized melanin is not reverted to untreated melanin by the reduction by ascorbate.

It was found that H_2O_2 decreases the radical concentration in melanins (Fig. 7). This result is consistent with the result that melanins are bleached by H_2O_2.[35]

The ESR signals of synthetic melanins could be reasonably described by assuming the presence of two kinds of quinhydrone type complex in melanins. To elucidate our hypotheses, further kinetics studies are required.

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