Reaction of Synthetic Melanins with Redox Reagents. II. ESR Spectra Using Flow Method

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Electron spin resonance (ESR) spectra of synthetic pyrogallol melanin showed a hyperfine structure at pH 13.0 under anaerobic conditions but did not under aerobic conditions. The outer signal defined in a previous paper was observed in the reactions with hexacyanoferrate(III) at pH 11.5 and with $NaIO_4$ at pH 13.0. The reaction with $NaIO_4$ indicated that the outer signal is splitted into two lines with hyperfine coupling constant (hfcc) of 0.41 mT. Another signal which is splitted into two lines with hfcc of 0.65 mT was observed in the reaction with $NaBH_4$ at pH 13.0. $Na_2S_2O_4$ solution showed ESR signal under anaerobic conditions and this signal disappeared by a large amount of melanins. The broad signal defined in a previous paper disappeared slowly by $Na_2S_2O_4$ but did not by $NaBH_4$. Time courses of ESR signal in reactions of melanins with redox reagents were shown up to 30 min after mixing.

In spite of many studies on melanins, their chemical structure has not been elucidated because of their insolubility and irregularity. Ultraviolet and visible absorption spectra revealed only a structureless band.[1-3] Infrared absorption spectra showed only C=O and O-H stretching bands.[1,2] X-ray diffraction invariably reveals a lack of crystallinity. The information about the chemical structure of melanins has been found to rely only on electron spin resonanc (ESR) spectroscopy. However, ESR spectra of melanins do not show generally hyperfine structure (hfs) although the hfs due to nitrogen nucleus in synthetic and natural pheomelanin,[4,5] or that due to nitrogen and hydrogen nuclei in pheomelanin from chicken feathers was observed.[6] The hfs in natural eumelanin or in synthetic β-(3,4-dihydroxyphenyl)alanine (dopa) melanin has not been observed. Our purpose has been to clarify the chemical structure around melanin radicals by the chemical modification of melanins using ESR technique.

In a previous paper, four kinds of ESR signal (main and broad signals under anaerobic conditions, and inner and outer signals under aerobic conditions) were observed in synthetic melanins.[7] It was not evident whether the outer signal is a single line or is splitted into two lines by hydrogen nucleus.

In this article, we report the time course of ESR spectra in the reactions of synthetic melanins with O_2, KO_2, $[Fe(CN)_6]^{3-}$, $NaIO_4$, $NaBH_4$, ascorbate, and $Na_2S_2O_4$ using a flow cell.

Experimental

Potassium superoxide, NaOD, D_2O, and 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy were obtained from Aldrich Chem. Co. and all other chemicals used were purchased from Wako Pure Chem. Industries and used without further purification.

Synthetic melanins used were the same in the previous paper.[7] The buffer solutions used were borate (17 mmol dm^{-3})-citrate (4.25 mmol dm^{-3})-phosphate (91.5 mmol dm^{-3}) buffer solution (pH 11.5) and KCl (50 mmol dm^{-3})-NaOH (40.8—132 mmol dm^{-3}) buffer solution (pH 12.0—13.0). KCl-NaOD (D_2O) buffer solution was used in a part of measurements.

Buffer solutions of redox reagents and of melanins were prepared freshly and separately under argon gas, and were mixed and introduced into an aqueous quartz flat cell (a JEOL LC-12 ESR cuvette, inner size 60 mm×10 mm×0.31 mm) with a micro feeder Model JP-V (Furue Science Co.). Mixing time was 2 s with a flow rate of 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 unless particularly noticed. ESR spectra in figures are depicted with a spectral gain of 1000 as a standard. The recording rate was 1.25 mT min^{-1}. Mn(II) diluted with MgO and diphenylpicrylhydrazyl were used as a g marker. Spin concentrations of melanins were estimated using a reference radical (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy) with a microwave power of 0.6 mW and a modulation amplitude of 0.5 mT. 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 and Discussion

The ESR signals of synthetic melanins under anaerobic conditions did not change their strength for 1 h in the all pH range examined.

The spin concentrations of pyrogallol and dopa melanins were estimated to be about 9.5 and 6.7×10^{18} spins g^{-1}, respectively. It follows that there is one spin per 500 and 600 monomers, respectively, if melanins are assumed to be derived from precursors by a loss of two hydrogen atoms. It should be noted that the main and broad signals contribute to these spin concentrations.

Reaction with O_2. Since oxygen was not soluble enough in buffer solutions to apply to the reaction using the flow method, the reaction of melanins with O_2 was followed after O_2-bubbling for 1 min. Figure 1 shows ESR spectra in the reaction of pyrogallol melanin with

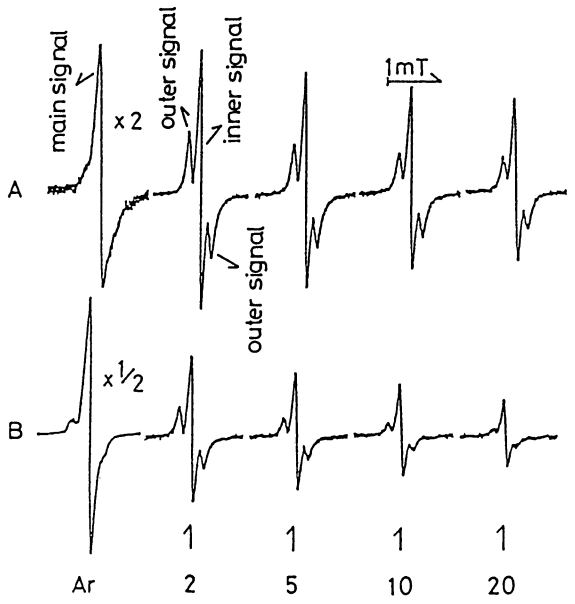


Fig. 1. ESR spectra in the reaction between O_2 and pyrogallol melanin (0.40 mg cm^{-3}). The number in the figure is time (min) after the cessation of O_2-bubbling for 1 min. A: pH 11.5. B: pH 13.0.

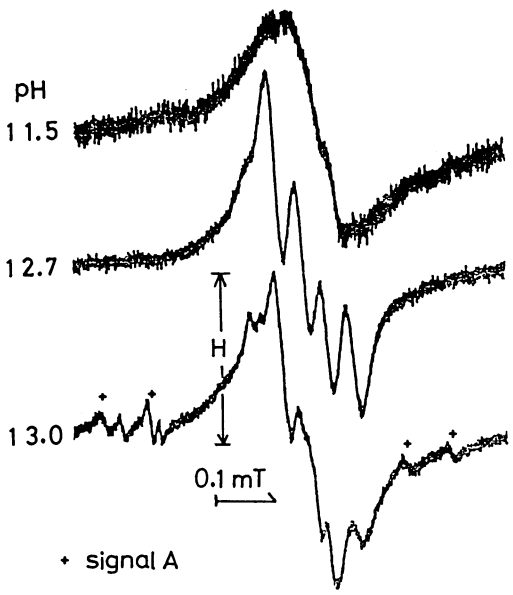
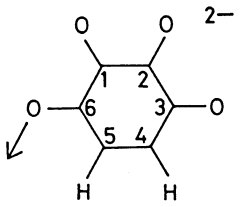


Fig. 2. Expanded ESR spectra of pyrogallol melanin in anaerobic solutions. Modulation width was 0.01 mT.

O_2 at pH 11.5 (A) and 13.0 (B). The inner and outer signals appeared by aeration. At pH 11.5, the line shape was maintained for 20 min after the cessation of O_2-bubbling but the both signal strengths were weakened gradually. This suggests that oxygen consumption by melanin continued for about 20 min in the solution, and that radical formation and destruction were occurred at the same time. The signal strength at pH 13.0 was about four times that at pH 11.5 under anaerobic conditions (Ar). Since the rates of oxygen consumption[8] and H_2O_2 formation[9] by melanin become larger with increasing pH, the radical formation may be finished at an early time after the cessation of O_2-bubbling and the radical destruction by H_2O_2 was occurred resulting in the rapid decrease of the signal strength by aeration at pH 13.0.

The ESR spectra of pyrogallol melanin showed a hyperfine structure under anaerobic (Ar) conditions at pH 13.0 but did not at pH 11.5. Figure 2 shows the expanded ESR spectra of pyrogallol melanin under anaerobic conditions. The line shape in KCl–NaOD–D_2O buffer solution was similar to that in KCl–NaOH–H_2O buffer solution. This indicates that the hyperfine structure is due to deprotonated radical(s). The main signal showed hyperfine structure at pH 12.7. Another signal (signal A in Fig. 2) was observed at pH 13.0. This signal has hfcc of 0.51 mT and 0.08 mT, and g value of 2.0038. These hfcc are similar to that of free pyrogallol anion radical (a_{5H}=0.54 mT, a_{4H}=a_{6H}=0.093 mT) in our buffer solutions. A possible structure of the radical contributed to the signal A is considered to be a following formula.



Where mark→means that radical is bound to large aryl groups and π-electrons are omitted. The g value of the signal A is different from that of the broad signal (2.0030). Therefore, the signal A is not the same to the broad signal. The line shape in the region, at which the main signal appeared, at pH 13.0 was different from that at pH 12.7. The line shape at pH 13.0 indicates that there are many radical species in pyrogallol melanin. The inner and outer signals under aerobic conditions did not show the hyperfine structure even at pH 13.0.

Figure 3 shows the microwave saturation of the ESR signals of melanins at pH 13.0 under anaerobic conditions. The saturation of the signal of pyrogallol melanin occured at microwave power lower than that of dopa melanin.

Reaction with KO_2. Figure 4 shows the ESR spectra of the reaction of melanins with superoxide at pH 13.0. The superoxide-induced radical[10] was not found at pH 13.0. The line shape of pyrogallol melanin during a continuous flow was similar to that under anaerobic conditions and its strength was weakened gradually with keeping its line shape after the flow was stopped. The ESR spectra of the reaction of pyrogallol melanin with KO_2 showed the outer signal at pH 12.8.[7] Since superoxide is unstable in the solutions at pH lower than 13, it

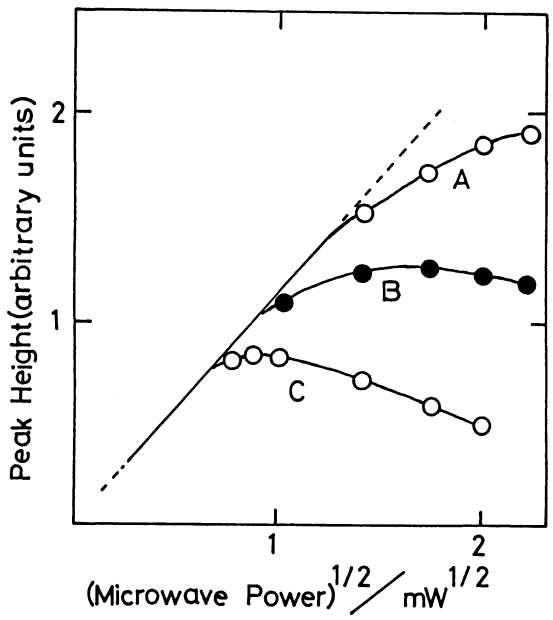


Fig. 3. Peak heights of ESR signals of melanins at pH 13.0 under anaerobic conditions as a function of microwave power. A: dopa melanin. B: total signal of pyrogallol melanin. C: H in Fig. 2.

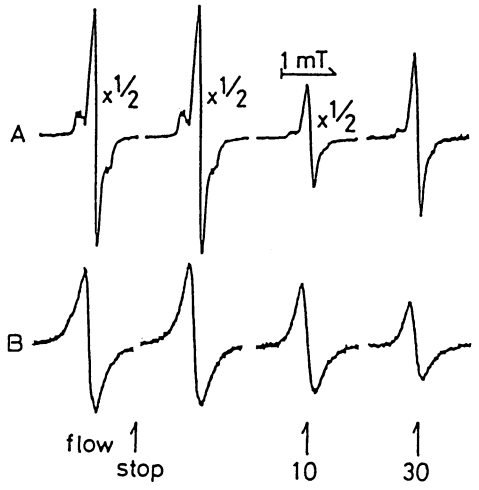


Fig. 4. Reaction between KO_2 (final concentration of 2.8 mmol dm^{-3}) and melanins (final concentration of 0.40 mg cm^{-3}) at pH 13. The number in the figure is time (min) after flow was stopped. A: pyrogallol melanin. B: dopa melanin.

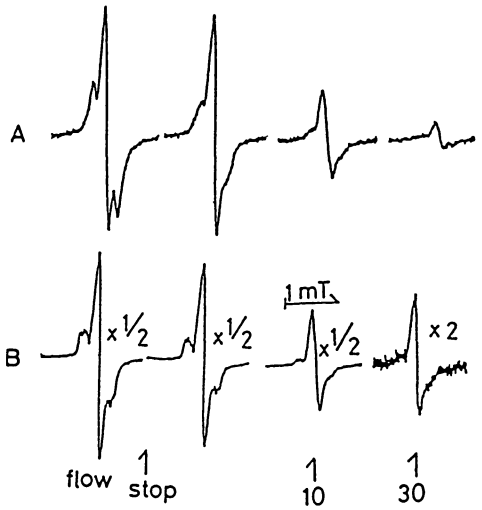


Fig. 5. Reaction between $K_3[Fe(CN)_6]$ (final concentration of 3.6 mmol dm^{-3}) and pyrogallol melanin (final concentration of 0.40 mg cm^{-3}). The number in the figure is time (min) after flow was stopped. A: pH 11.5. B: pH 13.0.

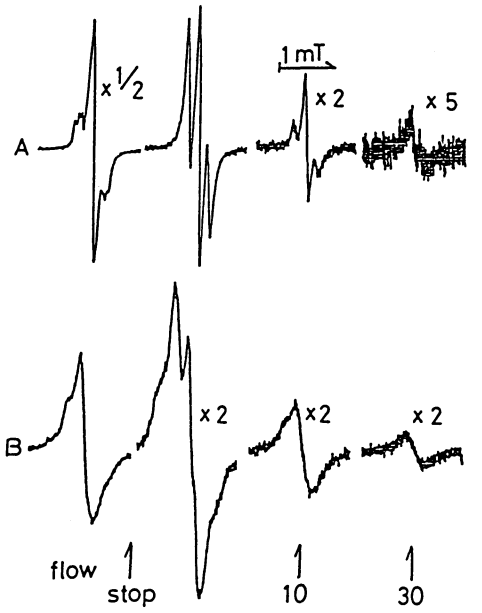


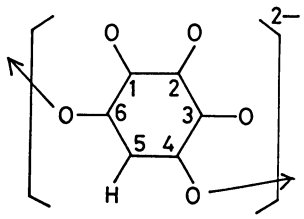
Fig. 6. Reaction between $NaIO_4$ (final concentration of 3.7 mmol dm^{-3}) and melanins (final concentration of 0.40 mg cm^{-3}) at pH 13. The number in the figure is time (min) after flow was stopped. A: pyrogallol melanin. B: dopa melanin.

is probable that oxygen molecule was generated in the solution of the reaction with KO_2 at low pH values.[7,10] The decrease rate of the ESR signal of dopa melanin was lower than that of pyrogallol melanin. Such a low reaction rate of dopa melanin compared with that of pyrogallol melanin was observed in the reactions with other reagents.

Reaction with $[Fe(CN)_6]^{3-}$. Figure 5 shows the ESR spectra of the reaction of pyrogallol melanin with hexacyanoferrate(III). At pH 11.5, the outer signal was observed during continuous flow even under anaerobic conditions. This indicates that the outer signal was formed not only by oxygen but also by hexacyanoferrate(III). The signal strength was decreased rapidly for 30 min after the flow was stopped. The melanin oxidized by hexacyanoferrate(III) at pH 6.8 showed a similar signal to that of untreated melanin but its signal strength was weaker than that of untreated melanin.[7] Since melanin is almost insoluble in a buffer solution at

pH 6.8, the flow method could not be applied to the reaction with hexacyanoferrate(III) at pH 6.8. However, the tendency of the ESR line shape between at pH 6.8 and 11.5 was similar to each other. At pH 13.0, the outer signal was not observed during continuous flow but the signal strength was decreased with a similar tendency at pH 11.5. It was found that hexacyanoferrate(III) oxidizes the radicals contributed to the main and broad signals, and signal A.

Reaction with $NaIO_4$. Figure 6 shows ESR spectra of the reaction of melanins with sodium periodate at pH 13.0. The spectra immediately after the flow was stopped showed large outer signals. It was found from the line shape that the outer signal of pyrogallol melanin is splitted into two lines of which hyperfine coupling constant is 0.41 mT and g value is 2.0038. A possible structure of the radical contributed to the outer signal is supposed to be a following formula. It was not clear



that the outer signal was generated by the oxidation by IO_4^- or by oxygen produced although oxygen bubbles were not observed in the reaction mixture. The outer signal of dopa melanin was also observed in the reaction with $NaIO_4$ and was splitted into two components with hfcc of 0.45 mT (Fig. 6B). The linewidth of the inner signal of dopa melanin was estimated to be 0.2 mT.

Reaction with $NaBH_4$. Figure 7 shows ESR spectra of the reaction of pyrogallol melanin with sodium borohydride. The main signal was rapidly disappeared after flow was stopped but the broad signal remained at pH 11.5. This agrees with the preceding data which were observed at 15 min after mixing in the pH range of 7.5—12.5.[7] At pH 13.0, a new signal which was splitted into two lines of which hfcc is 0.65 mT and g value is 2.0032 was observed. This signal is similar to the outer signal but not the same. The new signal is probably due to a radical which is reduced from quinone in a pyrogallol moiety, and is splitted into two lines by a hydrogen nucleus. The linewidth of a central signal after the flow was stopped at pH 13.0 was 0.25 mT and was similar to that of the broad signal of pyrogallol melanin (0.3 mT).

Reaction with Ascorbate. Time course of the ESR signal in the reaction of melanins with ascorbate was similar to that with KO_2 at pH 13.0 (data not shown). It was found that ascorbate quenches rapidly the signal A compared with KO_2 and that a large amount of ascorbate needs to eliminate the main signal at pH 13.0.

Reaction with $Na_2S_2O_4$. Sodium dithionite showed ESR signal in a powder form and in anaerobic solutions. The signal strength was not dependent on pH of solutions but the signal disappeared by air-bubbling. When a large amount of melanins was added to the

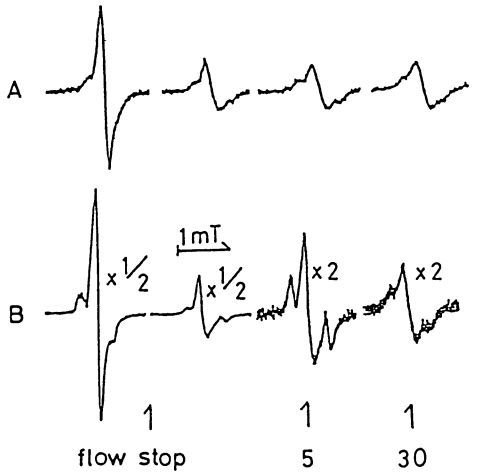


Fig. 7. Reaction between $NaBH_4$ (final concentration of 10.6 mmol dm^{-3}) and pyrogaloll melanin (final concentration of 0.40 mg cm^{-3}). The number in the figure is time (min) after flow was stopped. A: pH 11.5. B: pH 13.0.

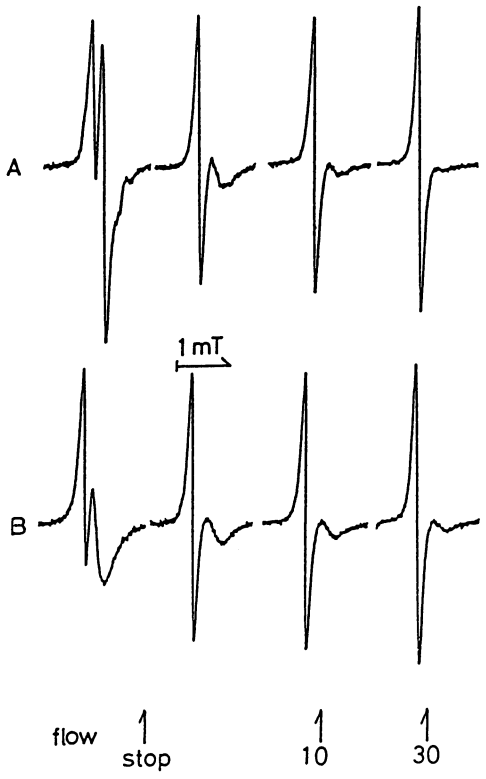


Fig. 8. Reaction between $Na_2S_2O_4$(final concentration of 4.6 mmol dm^{-3}) and melanins (final concentration of 0.40 mg cm^{-3}) at pH 13. The number in the figure is time (min) after flow was stopped. A: pyrogallol melanin. B: dopa melanin.

solution of dithionite, the ESR signal of dithionite disappeared. But when the amount of sodium dithionite was over 2 times of pyrogallol melanin by weight, the melanin signal disappeared and the dithionite signal remained. This indicates that the radical in dithionite is oxidized by melanin radical. Figure 8 shows the ESR spectra of the reaction of melanins with sodium dithionite at pH 13.0. The main and broad signals remained during a continuous flow. When the flow was stopped, the main signal disappeared rapidly and the broad signal weakened gradually. The broad signal was found to be reduced by dithionite. However, the broad signal of dopa melanin was decreased very gradually compared with that of pyrogallol melanin.

Conclusion. The result of the reactions of melanins with redox reagents at pH 13.0 led to the following conclusions:

(1) Oxygen molecule and $NaIO_4$ oxidized rapidly the radicals contributed to the main and broad signals and produced the inner and outer signals. But these signals produced were rapidly quenched. The quenching under aerobic conditions is considered to be due to H_2O_2 produced in the solutions.

(2) KO_2 and $[Fe(CN)_6]^{3-}$ oxidized the radicals contributed to the main and broad signals and any new signals were not observed at pH 13.0.

(3) $NaBH_4$ and ascorbate reduced the radical contributed to the main signal but did not reduce the radical contributed to the broad signal. $NaBH_4$ produced the new signal which is splitted into two lines with hfcc of 0.65 mT.

(4) $Na_2S_2O_4$ reduced the radical contributed to the main signal very rapidly and that contributed to the broad signal slowly.

(5) The rate of reaction of dopa melanin with redox reagents was small compared with that of pyrogallol melanin. It may be considered that indole fragments produced by the oxidation of dopa prevents the reactions.

The radicals which contribute to the broad and main signals were confirmed to be produced from different quinhydrone type complexes. The ESR spectra under anaerobic conditions at pH 13.0 suggest that the molecular structure of melanins is very complex. However, the chemical structure of some radicals may be clarified from their hyperfine structures.

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