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## Fluorescence Spectroscopy of Eumelanins\*

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**Abstract**—The fluorescence spectra from different forms of melanin, excited by an argon-ion laser at 488 nm wavelength were measured. The fluorescence intensity and maxima were found to be dependent on the melanin concentration. The location of the fluorescence maxima depends on the type of melanin and its environment. Solubilization of melanin markedly increases its fluorescence emission and it is postulated that an oxidative cleavage of the quinoid structure of the indole moiety present in the melanin is associated both with the solubilization of the pigment as well as with an enhancement of fluorescence.

### I. INTRODUCTION

**E**UMELANIN is a natural pigment [1] of diverse functional characteristics found in all phyla. Among others it is responsible for coloration of hair and skin and in the latter case it is also associated with a photo-protective function. Largely due to its insolubility, the eumelanin has been difficult to characterize in spite of many efforts in this direction [2]–[5]. A mild technique for melanin solubilization has been developed [6] offering a fresh approach to spectroscopic evaluation of melanins. Fluorescence spectroscopy is a method which can reveal changes in the chemical composition of a multicomponent system which may include melanin. The information gained from fluorescence measurement should allow us to improve our understanding of specific treatments of hair or skin. Fluorescence offers a new tool to probe melanin on a mo-

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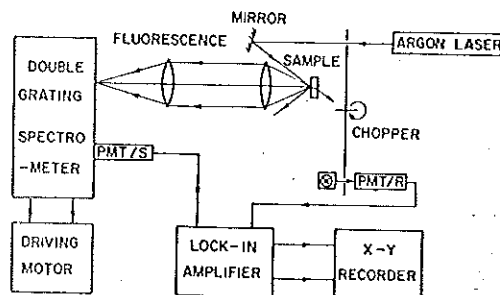


Fig. 1. Schematic diagram of fluorescence setup.

lecular level. This paper reports on the measurement of the fluorescence spectra of both intact and solubilized eumelanins.

### II. METHODS

#### A. Experimental Setup

The fluorescence measurements were performed using the experimental setup, shown in Fig. 1. An argon-ion laser operating at 100 mW was used as an exciting source at a 488 nm wavelength. The samples were excited on the first surface. The fluorescence was collected and passed into a SPEX double-grating spectrometer with automatic wavelength sweep. The fluorescence signal was measured by an RCA 7265-(S-20) photomultiplier tube (PMT). The PMT signal was detected by a lock-in amplifier (LIA). The signal was displayed as a function of wavelength on an X-Y recorder. The spectra were not corrected for spectral response of photodetector and spectrometer system. All measurements were performed at room temperature.

Fluorescence curves shown in this paper were taken with different sensitivities of detection used at different concen-

trations. To facilitate comparison of fluorescence signals, we selected the sensitivity range of the LIA of  $50 \mu\text{V}$  as a reference and introduce a multiplicative factor for each sensitivity range. The multiplicative factor for any sensitivity range of the LIA is thus given as a ratio of its sensitivity to the reference sensitivity of  $50 \mu\text{V}$

$$\mathcal{L} = \frac{\text{LIA}}{50 \mu\text{V}}$$

The values  $\mathcal{L}$  referring to particular conditions are given in the figure captions of all the graphs. As an example, one can notice that for LIA sensitivities of  $200 \mu\text{V}$  and  $100 \text{ mV}$ , the factor  $\mathcal{L}$  stands for 40 and 2000, respectively. Thus, in order to compare any two fluorescence signals one should read them first in terms of the units of their graphs and then multiply the results by relevant sensitivity factors ( $\mathcal{L}$ ).

### B. Samples

1) *Human Hair Melanin*: Melanin pigment was extracted from black human hair by the method of Laxer [7]. The samples were prepared as mixtures of various concentrations of melanin with KBr powder and polycrystallized in a laboratory press in the form of pellets. The solubilized human hair melanin was prepared by gentle oxidation with alkaline hydrogen peroxide. These samples were prepared as aqueous solutions of concentrations  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $0.5 \times 10^{-4}$ , and  $10^{-5}$  M. The molarity of these solutions was calculated (for reference purposes) using the molecular weight of 150 for the hypothetical precursor of melanin 5,6-dihydroxyindole (DHI).

Solid KBr samples of the solubilized human hair melanin were also prepared to compare their fluorescence spectra to those of the solid KBr samples of the unsolubilized human hair melanin.

2) *Synthetic Melanin*: The synthetic melanin samples were produced from 5,6-dihydroxyindole (DHI) by its autooxidation at pH 9.7 (5,6-diacetoxyindole was used as DHI precursor). The melanin was soluble in 0.1 N NaOH, but not in water. A water soluble derivative was prepared by solubilization of the synthetic melanin using 0.5 percent  $\text{H}_2\text{O}_2$  at pH 10. The KBr pellets of both melanins were prepared at different melanin concentrations. Similar dilutions were obtained also from the solutions of these melanins.

3) *Squid Melanin*: Melanin removed from squid "ink" sacks was solubilized with 0.25 percent  $\text{H}_2\text{O}_2$  at pH 11.25 (adjusted by using NaOH) for 1 h. The reaction was quenched by addition of 1 N HCl to lower the pH to 9.5, and addition of about 5 mg of platinum black. The platinum black was then removed by centrifugation and the sample dialyzed (MWCO 3,500) against  $\text{H}_2\text{O}$ . At this point, the concentration of melanin was 0.1 percent per weight (marked as the concentration 100 percent in the description of the corresponding graph). Other samples were prepared from that initial one by sequential dilutions in water to the concentrations 1:1,  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$  per weight.

## III. RESULTS AND DISCUSSION

### A. Fluorescence of Human Hair Melanin

The fluorescence spectrum of the intact pigment in KBr is shown in Fig. 2. Only a very weak fluorescence is obtained

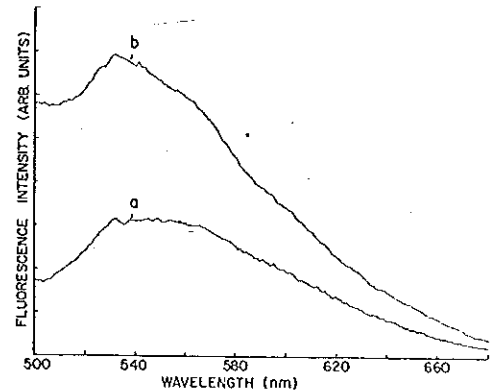


Fig. 2. Fluorescence of human hair melanin in KBr for two samples at concentration of  $10^{-2}$  M, taken with different sensitivities: (1)  $\mathcal{L} = 4$ , (2)  $\mathcal{L} = 2$ .

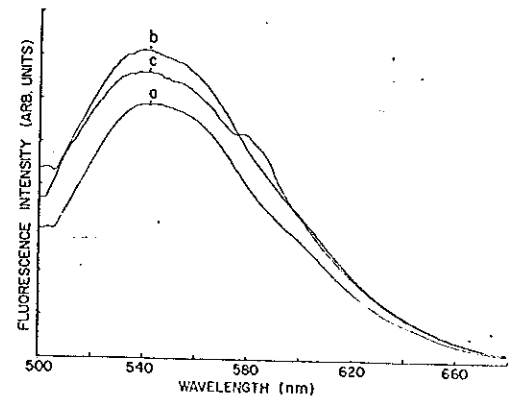


Fig. 3. Fluorescence spectrum of solubilized human hair melanin at high concentrations: (a)  $10^{-2}$  M,  $\mathcal{L} = 200$ ; (b)  $10^{-3}$  M,  $\mathcal{L} = 40$ ; (c)  $10^{-4}$  M,  $\mathcal{L} = 4$ .

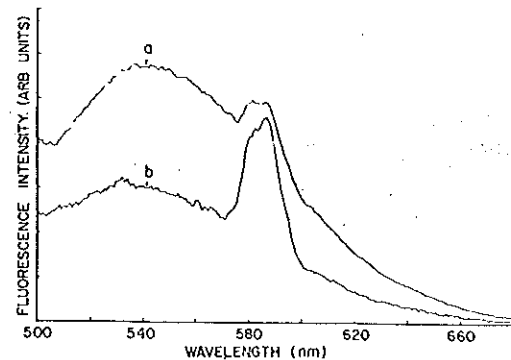


Fig. 4. Fluorescence spectrum of solubilized human hair melanin at low concentrations: (a)  $0.5 \times 10^{-4}$  M,  $\mathcal{L} = 2$ ; (b)  $10^{-5}$  M,  $\mathcal{L} = 1$ .

even for concentration as high as  $10^{-2}$  M. The weakness of the signal must have been the cause for an earlier report found in the literature [8] that melanin does not fluoresce.

The process of solubilization brings about a dramatic increase in the intensity of fluorescence. A yellowish fluorescence of the solubilized melanin is strong enough to be readily seen by the naked eye, even against the background of the blue argon-laser exciting light. The fluorescence spectra are displayed in Fig. 3 and Fig. 4. The maximum of a typically shaped fluorescence curve for these samples is located at 542 nm for  $10^{-3}$  M and lower concentrations. However, for higher concentration

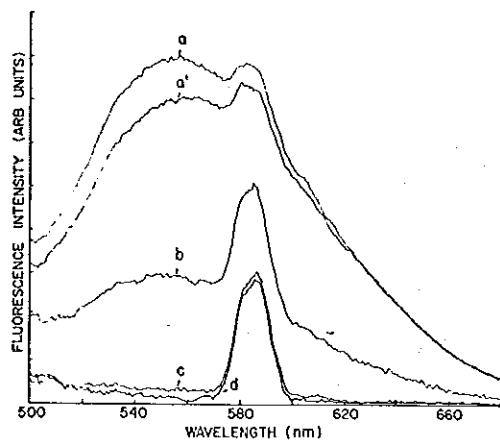


Fig. 5. Fluorescence of synthetic melanin in solution of 0.1 N NaOH, (a)  $10^{-1}$  M,  $\lambda = 2$ , and for subsequent water dilutions of: (b)  $10^{-2}$  M,  $\lambda = 1$ ; (c)  $10^{-3}$  M,  $\lambda = 1$ ; (d)  $10^{-4}$  M,  $\lambda = 1$ .

of  $10^{-2}$  M solution, the maximum shows a shift of about 3 nm towards shorter wavelengths; a result which has been carefully confirmed. For the lowest concentrations ( $10^{-5}$  M and  $0.5 \times 10^{-4}$  M), one can observe the typical shape of fluorescence curves on which a Raman scattering signal, at 585 nm, coming from interaction of the laser light with water solvent molecules, is superimposed. This effect is noticeable at concentration of  $10^{-4}$  M.

Seeking an explanation for the increase in fluorescence of melanin upon solubilization one might relate it to the recent observation of chemi- and photoinduced chemiluminescence of melanins [9]. The mechanism postulated in the latter case involved primarily oxidative cleavage of the carbon-carbon bond between quinoid carbonyls of the 5,6 dihydroxyindole moiety, which is generally considered to be the building block of eumelanins. This is also the most likely site that yields to the peroxide attack in the process of melanin solubilization. One would have to suggest, therefore, that chemiluminescence associated with the oxidative cleavage reactions is only a weak precursor to the formation of a permanent and strong fluorophore.

### B. Fluorescence of Synthetic Melanin

The fluorescence of the synthetic melanin in solution of 0.1 N NaOH, is very weak (Fig. 5) even for the highest concentration of  $10^{-1}$  M. The fluorescence spectra are noticeable but weaker than the Raman scattering from water for the  $10^{-2}$  M concentration. Negligible fluorescence was observed for concentrations of  $10^{-3}$  M and lower. Significant scatter of results indicated by the two fluorescence curves marked (a) (a') in Fig. 5 gives an idea of the reproducibility of results taken at this sensitivity level ( $\lambda = 2$ ).

In a chemical sense there is a strong similarity between the synthetic melanin and that which is derived from human hair. Both presumably contain intact indole residues and both show very weak fluorescence either in KBr or in aqueous solution. The process of solubilization increases the fluorescence many fold and the similarity of emission characteristics suggest common nature of the fluorophores.

The fluorescence of the solubilized derivative is shown in Fig. 6. Comparing spectrum (d) from Fig. 6 with that marked

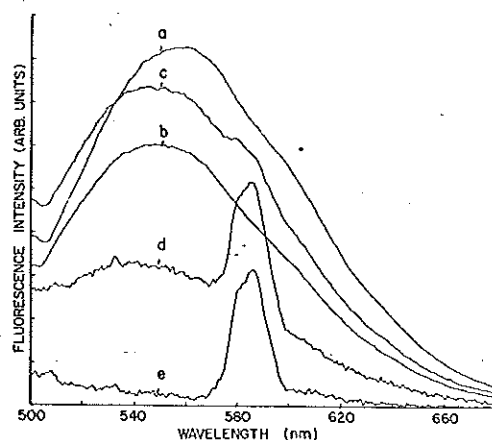


Fig. 6. Fluorescence of the solubilized derivative of synthetic melanin in water: (a)  $10^{-2}$  M,  $\lambda = 40$ ; (b)  $10^{-3}$  M,  $\lambda = 40$ ; (c)  $10^{-4}$  M,  $\lambda = 4$ ; (d)  $10^{-5}$  M,  $\lambda = 1$ ; (e)  $10^{-6}$  M,  $\lambda = 1$ .

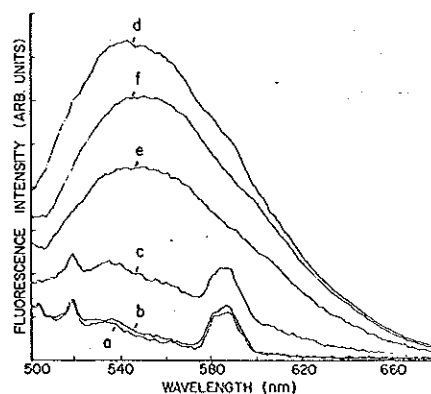


Fig. 7. Fluorescence of water soluble squid melanin for various concentrations (per weight) and relevant detection sensitivities; (a) pure solvent (water),  $\lambda = 2$ ; (b)  $10^{-3}$  per weight,  $\lambda = 2$ ; (c)  $10^{-2}$  per weight,  $\lambda = 2$ ; (d)  $10^{-1}$  per weight,  $\lambda = 4$ ; (e) "1:1" per weight,  $\lambda = 20$ ; (f) "100 percent" weight,  $\lambda = 20$ .

as (b) in Fig. 5, one can notice the same fluorescence signal from the  $10^{-5}$  M solution of the melanin derivative, as from the  $10^{-2}$  M solution of the synthetic melanin. Also, comparing fluorescence signals for the same concentrations of  $10^{-2}$  M (curve (b) in Fig. 5 with the curve (a) in Fig. 6) one can confirm a 100 times increase in the fluorescence yield, which clearly results from the solubilization process.

The emission intensity from hair and synthetic melanin was measured and found to depend linearly on the excitation intensity, thus eliminating a two-step phosphorescence mechanism. The observed emission arises from a fluorescence process. This has been confirmed by the ultrafast recombination time in the nanosecond regime [10].

### C. Fluorescence of Solubilized Squid Melanin

Further confirmation of the structural similarity of the diverse melanins and of the alike chemistry of the solubilization process can be found in the fluorescence spectra of solubilized squid melanin.

The spectral characteristics of these samples (Fig. 7) are similar to those for the water soluble human hair melanin (Figs. 3 and 4). The spectrum of the sample at the lowest concentration ( $10^{-3}$  per weight) seems to be exactly the same as that of

pure water solvent, indicating outstanding Raman scattering of the argon-laser light ( $\lambda = 488$  nm) in water at  $\lambda = 585$  nm. It is important to notice that the initial parts of all fluorescence curves presented herein might be misleading since the fluorescence signal measured at the vicinity of  $\lambda = 500$  nm is strongly affected by the argon-laser exciting light of  $\lambda = 488$  nm scattered within the spectrometer. This is why all local peaks at this area, including those accidentally coinciding with Raman scattering at low frequency below 520 nm should be regarded as artifacts rather than valuable informations. For the concentration  $10^{-2}$  per weight, there is a weak fluorescence signal which is comparable in intensity to the Raman scattering signal. For this concentration, the fluorescence can be observed through a Corning 3-67 yellow filter which cuts off the laser exciting light. For higher concentrations, the fluorescence signal is well distinguishable by the naked eye. For the highest concentrations ("100 percent" and "1:1"), the wavelength of the fluorescence maximum is about 550 nm. For lower concentrations ( $10^{-1}$  per weight and  $10^{-2}$  per weight), it is shifted about 7 nm towards shorter wavelengths. This effect is opposite to that observed in the case of the hair melanin, where higher concentration shifts to the blue.

#### IV. CONCLUSION

While the natural or synthetic melanins show only very weak fluorescence, their soluble derivatives prepared by mild oxidation with alkaline peroxide exhibit strong fluorescence emission. Such fluorescence excited by 100 mW argon-ion laser light at 488 nm is in most cases well seen by the naked eye, and precisely detectable by a double-grating spectrometer and lock-in phase-detection. The fluorescence response signal indicates significant concentration-dependent changes in its intensity and its maximum wavelength. The fluorescence-maximum wavelength is related to the type of melanin used and to the way samples are prepared. For the samples of solubilized human hair melanin in water, the fluorescence-maximum wavelength indicates a shift of about 3 nm towards longer wavelengths when the concentration changes from  $10^{-3}$  to  $10^{-4}$  M, while for solubilized squid melanin samples, the corresponding shift stands for about 7 nm towards shorter wavelengths as we decrease the concentration by 10-fold. It was also shown by Gallas [11] that modifications of melanin, such as a change in its ionization, seem to be well detectable through measurements of its fluorescence. The solubilization of melanin most probably arises from the oxidative cleavage of the quinoid ring of the indole moiety that is the mainstay of the melanin structure. We hypothesize that it is the products of oxidative transformation of indole residues that are the principal fluorophores involved in fluorescence of eumelanin.

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L. J. Wolfram, photograph and biography not available at the time of publication.

Robert R. Alfano, for a photograph and biography, see this issue, p. 1342.