Absorption Mechanisms of Human Melanin in the Visible, 400–720 nm

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In this paper we propose that human melanin absorbs visible radiation through two distinct mechanisms: one that is in effect over the entire visible range and is linear in wavelength, and a second one that is evident at wavelengths in the range 400–500 nm and is exponential in frequency. These mechanisms are apparent in all human diffuse reflectance spectra that we have collected. We show that the absorber is the same in all human volunteer skin samples. By studying the diffuse reflection spectra of DOPA-melanin in solution and DOPA-melanin in powder form, we find that we can correlate the absorption mechanisms, one with melanin in solution (a low molecular weight form) and the other with melanin in powder (a high molecular weight form). Therefore, we propose that melanin exists in two distinct states.

This model is of biologic significance, as it provides a reasonable interpretation for the diffuse reflection spectra obtained from delayed pigment (UVB-induced) and immediate pigment (UVA-induced). Delayed pigment appears as an increase of both forms of melanin (neomelanogenesis), whereas immediate pigment appears as an increase in the higher molecular weight form with a commensurate decrease in the lower molecular weight form: the two mechanisms change independently of each other. Finally, we show that we can distinguish spectroscopically between the delayed pigment and the immediate pigment. *J Invest Dermatol 89:384–388, 1987*

In recent years the absorption properties of DOPA (3,4-dihydroxy-phenylalanine)-melanin as well as extracted natural melanins have been widely studied [1–4], with reasonably good agreement among the various reports. In these studies, the spectra have been obtained by putting the melanin in solution and obtaining the absorbance by standard spectrophotometric techniques [4]. The absorption spectrum of melanin is interesting, in that it shows no characteristic absorption bands in the ultraviolet or the visible that could be used for identification. It has been proposed [2], and experimentally confirmed [1], that melanin behaves as an amorphous semiconductor when powder melanin is formed into pellets.

It has been shown [5] that the absorption spectrum of human melanin in skin in vivo in the wavelength range 620–720 nm is a linear function of the wavelength. It has also been shown [6] that it is the same absorber for all volunteers tested. Furthermore, it has been determined that the slope of the straight line is a sensitive indicator of the pigment level in the skin as it is perceived by the eye.

This study is concerned with the absorption properties of human melanin in skin in vivo over the entire visible range, 400–720 nm. The absorbance of melanin as a function of wavelength has been interpreted in terms of different mechanisms of absorption. Correlations have been explored between the physical mechanisms proposed and established biologic processes.

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Abbreviations:
DPD: delayed pigment darkening
IPD: immediate pigment darkening
UVA: ultraviolet radiation (320-400 nm)
UVB: ultraviolet radiation (280-320 nm)

MATERIALS AND METHODS

In Vivo Measurements Diffuse reflectance spectra were obtained from 35 vitiligo and 24 normal volunteers. Informed consent was obtained from all volunteers before measurements were made. In the case of the vitiliginous volunteers, two measurements were conducted: one on a vitiliginous involved skin area and the other on an adjacent normally pigmented skin site. The distance between the involved and noninvolved areas was 50 mm maximum. The sites were photographed, on a one-to-one scale, immediately after the measurements were completed. All measurements were conducted on the inside surface of the forearm. The eye color of all but one volunteer was dark brown.

Diffuse reflectance spectra were obtained from an area on the upper back of a volunteer who had been irradiated on one side with 20 J/cm^2 of UVA and on the other side with 150 mJ/cm^2 of UVB. The area which had been irradiated with UVA was assessed immediately post irradiation, to assess the immediate pigment darkening reaction (IPD). The area which had been irradiated with UVB was measured seven days later to assess the delayed pigment darkening raction (DPD).

The instrument used for the measurements has been described elsewhere [5,7].

In Vitro Measurements Diffuse reflectance spectra were collected from solid melanin samples using the same instrument. Enzymatic eumelanin was given to us by Dr. Miles Chedekel and DOPA-melanin in aqueous solution was given to us by Dr. R. Anderson. A solid solution was made with Al_2O_3 as the solvent, and the solid melanin as the solute. The mixture was ground together for 45 min with mortar and pestle until it was uniform. It was then placed in a black cup (4 mm depth, 25 mm in diameter)

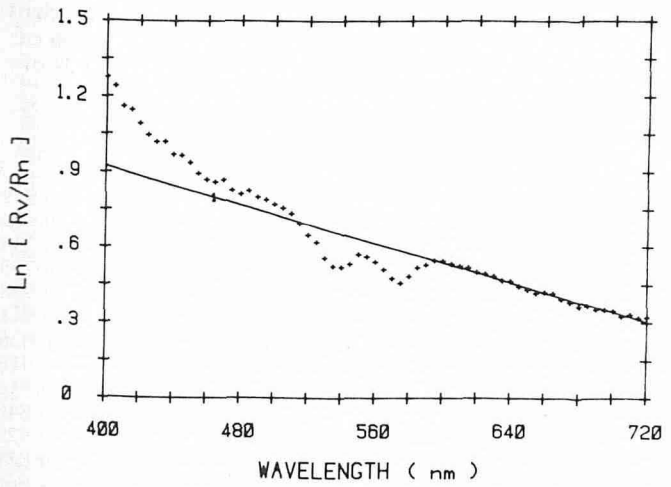

Figure 1. A typical absorption spectrum, or apparent absorbance, of human melanin in vivo versus wavelength in nanometers. This is accomplished by calculating the logarithm (base e) of the ratio of the remitted intensity from vitiliginous to normal skin, at adjacent sites of the same volunteer. The straight line represents the best fit through the experimental points in the range 620–720 nm. The correlation coefficient for the straight line is 0.982.

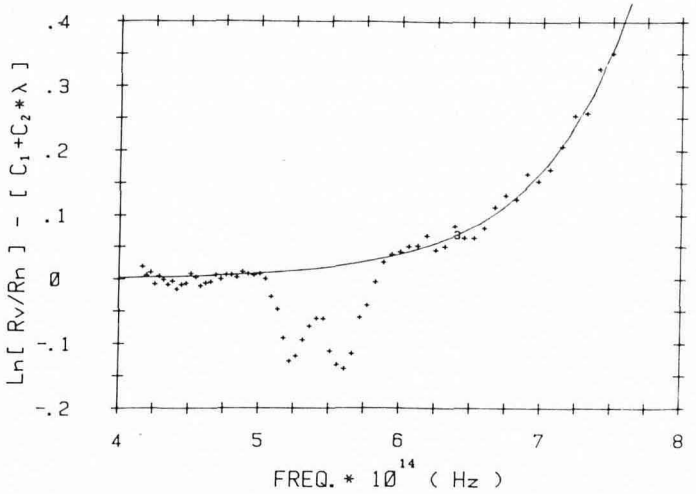

Figure 2. The deviation of the experimental points in *Fig 1* from the straight line fit are plotted versus the frequency of the light in Hz. The exponential curve is fitted to the experimental points in the range 400–500 nm ($7.50–6.00 \times 10^{14}$ Hz). The two resonance absorption peaks belong to the 542 and 577 nm absorption maxima of oxyhemoglobin.

and the diffuse reflectance spectrum was obtained by bringing the instrument probe against the cup. Absorption spectra of DOPA-melanin in aqueous solution were obtained on the above instrument and on a diode-array absorption spectrophotometer (Hewlett-Packard, Model 8450A). The two instruments gave identical results.

RESULTS

In Vivo Measurements The apparent absorbance of human melanin in vivo can be obtained by calculating the difference in the diffuse reflectance spectra from vitiliginous and normal skin of the same volunteer. Thus, by using vitiliginous skin as the reference, we were able to obtain the absorption spectrum of human melanin in vivo. It has been hypothesized that the absorption spectrum of melanin should be dominated by scattering, as it shows no characteristic absorption resonances in the visible or the ultraviolet [2]. The mechanisms that would be applicable in this spectral range would be Mie and Rayleigh scattering; however, it has not proved possible to fit our data with any linear combination of these. We therefore decided to develop an empirical method of analyzing the data while looking for an ab initio type of calculation that might lead us to a reasonable interpretation of the results.

We have attempted to fit the experimental data with the best mathematical curve that we might find and then attempt to understand what this type of fitting might mean. The spectral absorbance of human melanin cannot be fitted with a single curve. We therefore attempted a two component fit. One component is a straight line, in wavelength, and is apparent at wavelengths longer than 620 nm (Fig 1). We do not assume that the mechanism of absorption is active over this spectral range only; on the contrary, we assume that it is in effect over the entire visible range. We have shown [5,6] that this type of analysis is applicable to vitiliginous volunteers' skin, as well as that of normal volunteers. We have also shown that we are dealing with the same absorber in all these cases. The experimental curves for the absorption of melanin in vivo show a substantial deviation from the straight line, as can be observed in Fig 1.

The deviations of the experimental points from the straight line are then plotted as a function of frequency. We then perform a mathematical fitting of the points by a calculated curve. The type of functional relation that yields the best fit to the points is an exponential (Fig 2). These relations have been arrived at by trial and error as well as by looking for dependences on wavelength and frequency that made some physical sense. Table I gives the values of the parameters C_3 and C_4 for the exponential fit for all the vitiliginous volunteers. The parameters C_3 and C_4 are the constants of the exponential fit as follows

$$\text{Deviation} = C_3 \exp [C_4 \nu]$$

wherein ν is the frequency of light in Hz $\times 10^{14}$.

In the case of normal volunteers, we apply the same analysis, using as a reference 100% amelanotic skin of the whitest vitiliginous volunteer. The deviation of the experimental points from the straight line can be well represented by an exponential in frequency for more than 80% of the cases studied. The remaining cases were either very dark Africans or cases in which hemoglobin absorptions interfered strongly with the analysis. Northern Europeans gave a slope of the straight line, which is very small and a weak exponential deviation, whereas for Mediterranean-type skin the slope of the straight line becomes larger with a significant deviation from the straight line at short wavelengths. Dark Africans show a straight line absorption of a still steeper slope over the entire visible range, as does solid melanin (without the exponential deviation) (see Fig 2 of [8]). The parameters C_3 and C_4 for all the normal volunteers are tabulated in Table II.

In order to estimate the newly formed pigment in the case of IPD and DPD, we compare an area that has obvious hyperpigmentation with one that is normally pigmented and adjacent to the first. The curves that are obtained by comparing hyperpigmented skin to adjacent normal skin should yield the spectral apparent absorbance of IPD and DPD. Figure 3 shows two such curves, one from IPD and the other from DPD. The curves presented here are representative of many (>12) that we have obtained in both the case of IPD induced by UVA and DPD produced by UVA or UVB: that extensive data will be presented in a later report.

It can be noticed from Fig 3 that the apparent absorbance of IPD and DPD are remarkably similar in the long wavelengths ($\lambda > 600$ nm). The two curves are though very different in the short wavelengths ($\lambda < 500$ nm). As a matter of fact, the apparent absorbance in the case of DPD appears very similar to the spectrum of human melanin (neomelanogenesis), whereas the apparent absorbance of IPD shows a negative deviation from the straight line fit at wavelengths shorter than 500 nm. Thus, we find that the apparent absorbances of IPD and of DPD are characteristically

Table I. Coefficients C_3, C_4, and the Correlation Coefficient (R^2) for the Exponential Fit[a] in Frequency, of the Deviation of the Experimental Points From the Straight Line Fit, for 35 of the 43 Vitiliginous Volunteers Measured

Volunteer Number	C_3	C_4	R^2
1	5.27E − 04	0.95	0.969
2	3.07E − 05	1.32	0.987
3	4.67E − 09	2.43	0.965
4	2.33E − 06	1.67	0.908
5	1.05E − 04	1.17	0.979
6	2.08E − 04	1.09	0.988
7	1.91E − 06	1.73	0.985
8	6.70E − 07	1.83	0.896
9	6.14E − 05	1.11	0.914
10	5.18E − 10	2.82	0.805
11	5.46E − 15	4.16	0.744
12	8.09E − 04	0.79	0.869
13	1.98E − 07	1.93	0.934
14	7.94E − 13	3.65	0.961
15	4.29E − 04	0.98	0.903
16	2.50E − 07	1.93	0.980
17	1.50E − 03	0.66	0.644
18	4.37E − 04	0.93	0.780
19	2.16E − 15	2.29	0.861
20	2.33E − 04	0.91	0.867
21	2.44E − 04	1.06	0.979
22	3.68E − 05	1.37	0.975
23	1.04E − 04	1.14	0.781
24	5.05E − 06	1.48	0.958
25	3.37E − 08	2.00	0.980
26	1.81E − 06	1.73	0.969
27	1.45E − 05	1.41	0.869
28	4.52E − 19	5.42	0.749
29	1.88E − 13	3.77	0.811
30	1.16E − 15	4.54	0.825
31	3.25E − 09	2.40	0.998
32	2.38E − 14	4.11	0.741
33	9.61E − 11	3.09	0.940
34	3.89E − 04	0.90	0.923
35	3.56E − 05	1.13	0.951

[a]The exponential fit represents a mechanism of absorption of human melanin that acts in addition to the mechanism that shows a linear dependence in wavelength and is acting over the entire visible range.

Table II. Coefficients C_3, C_4, and the Correlation Coefficient (R^2) for the Exponential Fit in Frequency, of the Deviation of the Experimental Points from the Straight Line Fit, for 24 of the 28 Normal Volunteers Measured

Volunteer Number	C_3	C_4	R^2
1	5.88E − 11	2.89	0.519
2	1.03E − 07	2.13	0.843
3	5.06E − 09	2.50	0.934
4	3.50E − 08	2.22	0.795
5	7.92E − 11	2.95	0.938
6	1.70E − 06	1.69	0.911
7	2.80E − 14	3.99	0.836
8	2.34E − 10	2.83	0.916
9	8.51E − 10	2.68	0.916
10	2.23E − 13	3.73	0.848
11	1.44E − 10	2.89	0.929
12	6.67E − 08	2.02	0.839
13	9.97E − 10	2.64	0.866
14	4.02E − 08	2.14	0.937
15	2.92E − 12	3.41	0.957
16	2.06E − 11	3.13	0.764
17	4.70E − 09	2.47	0.936
18	5.20E − 07	1.78	0.901
19	1.85E − 15	4.44	0.848
20	1.31E − 13	3.66	0.957
21	1.18E − 09	2.58	0.949
22	1.10E − 17	4.98	0.958
23	1.89E − 09	2.58	0.808
24	1.87E − 16	4.56	0.758

The above parameters are arrived at by comparing the reflectance spectrum from each normal skin with that of a 100% amelanotic skin after each curve is corrected for the instrument function.

different to the extent that one could predict whether one is dealing with IPD or DPD simply by obtaining a diffuse reflectance spectrum from adjacent areas of hyperpigmented and normal skin.

In Vitro Measurements The absorption spectrum of human melanin in vivo as well as the spectra of solid melanin in solid solution and DOPA-melanin in aqueous solution are shown in Fig 4. Upon close inspection, it can be noticed that the absorption spectrum for human melanin is a curve from 400–600 nm, which becomes a straight line at wavelengths longer than 620 nm. A line is drawn that represents the best mathematical fit to the points at wavelengths longer than 620 nm. The absorption spectrum for DOPA-melanin in solution is not a straight line over the entire visible range, but can be represented by a straight line at wavelengths longer than 620 nm, whereas the apparent absorbance of melanin in powder form is a straight line over the entire visible range.

DISCUSSION

It has been shown [5] that we are dealing with the same absorber in all the vitiliginous and normal volunteers. This was accomplished by plotting the C_1 coefficient of the straight line fit against the C_2 coefficient. The fact that we obtained a very strong correlation between these two coefficients indicated that we were dealing with one and the same absorber in all the individuals studied. This statement is equivalent to saying that all the straight lines have an isosbestic point.

It has also been determined [8] that the slope of the straight line that best represents the apparent absorbance is a sensitive indicator of the pigment level in the skin as it is perceived by the eye. The question that is raised at this point is: Are the coefficients C_3 and C_4 correlated?

In Fig 5 the plot of $-\ln(C_3)$ versus C_4 for the vitiliginous

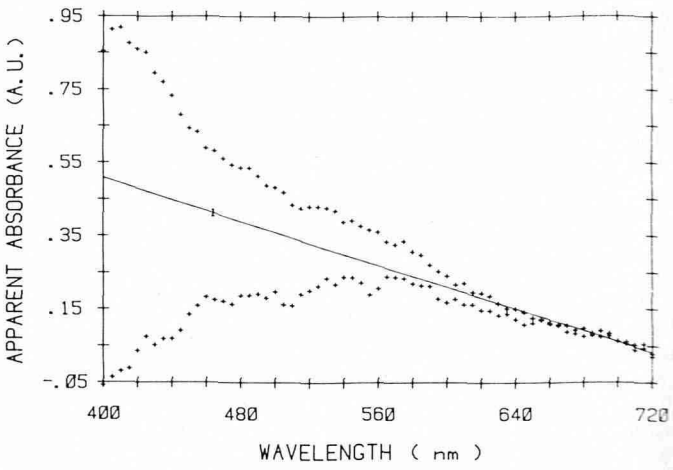

Figure 3. The apparent absorbance of delayed pigment darkening (DPD), generated by UVB (*upper curve*) and immediate pigment darkening (IPD) generated by UVA (*lower curve*) on the same volunteer. These curves are obtained by comparing the hyperpigmented lesion with adjacent normally pigmented skin. The DPD curve is concave upwards and the IPD curve is concave downwards at wavelengths shorter than 620 nm. The curves are similar at wavelengths longer than 620 nm.

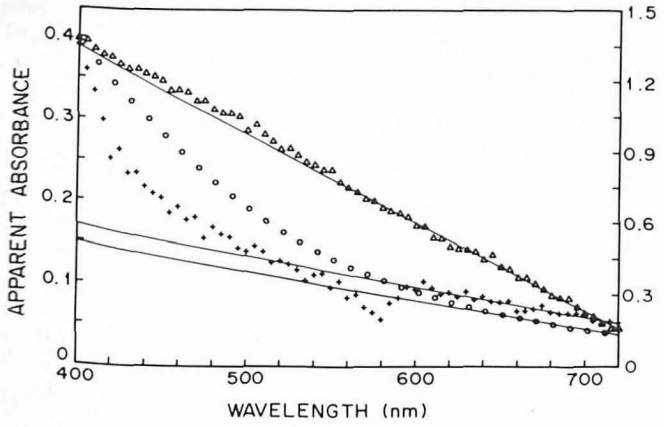

Figure 4. The apparent absorbance of human melanin in vivo and of melanin powder, as well as the absorbance of DOPA-melanin in solution (*right axis*) versus wavelength; *open triangles,* melanin powder; *plus signs,* human melanin; *open circles,* DOPA-melanin in solution. All curves are well approximated by straight lines at wavelengths longer than 620 nm.

volunteers and for the normal volunteers is indicated. It can be seen that these coefficients are very strongly correlated. As a matter of fact, the same correlation exists between the coefficients for the vitiligo as well as for the normal volunteers. We therefore conclude that we are dealing with one and the same absorber in the cases of vitiligo as well as in the cases of the normal volunteers, in the range of 400–500 nm. We have thus found that there exists a correlation between the coefficients that describe the straight line fit in wavelength in the range 620–720 nm, as well as between the coefficients that describe the exponential fit in the frequency, for the wavelength range 400–500 nm. It should be noted that the exponential deviation from the straight line depends strongly on our choice of coefficients C_1 and C_2. We have tried varying these parameters and have determined the variation in the exponential coefficients, we found that the value of the coefficients does change, however, the correlation between $-\ln C_3$ and C_4 remains the same. The next question to consider is whether there exists any correlation between the coefficients of the linear fit and the coefficients of the exponential fit.

In Fig 6, we show the plot of C_2 (the coefficient of the straight

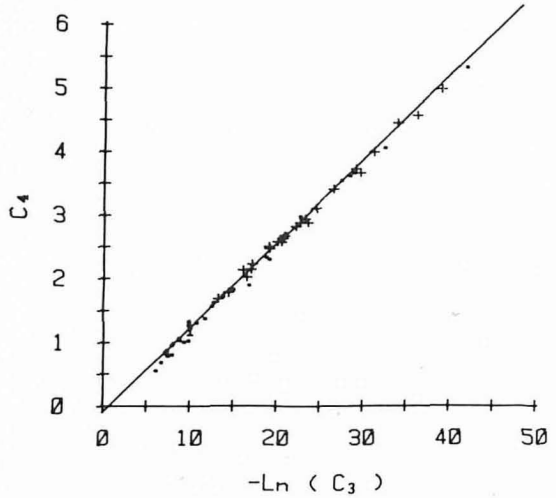

Figure 5. The coefficient C_4 of the exponential fit versus the logarithm, base *e*, of the other coefficient C_3. The plot shows the strong correlation between these two coefficients. *Dots,* vitiliginous volunteers; *plus signs,* healthy volunteers.

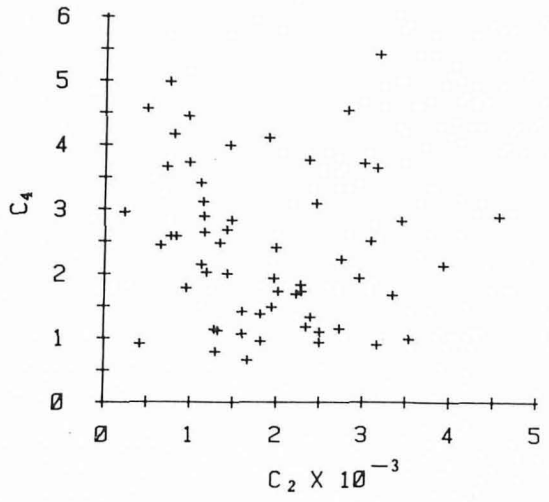

Figure 6. The coefficient C_4 of the exponential fit in frequency versus the coefficient C_2 of the linear fit in wavelength. The plot shows that no correlation exists between these two coefficients and, therefore, between the processes that they describe.

line fit) versus C_4 (the coefficient of the exponential fit). Figure 6 indicates no correlation between the two mathematical fits of the experimental data. We therefore propose that these represent two distinct mechanisms of absorption for human melanin in the range 400–720 nm. Considering the absorption spectra of DOPA-melanin in powder and in solution (Fig 4) we note that human melanin behaves as the powder and this behavior is evident at wavelengths 620–720 nm; and it deviates from the straight line just as the liquid does for wavelengths shorter than 500 nm. Based on these observations we further conclude that human melanin exists in two distinct forms, at least. One that is like a powder, i.e., large molecular aggregates or a high molecular weight form, and another that is like a liquid, i.e., a low molecular weight form. The terms high molecular weight and low molecular weight, in this context, are not used in an absolute sense, as we have made no attempt to classify extracted human melanin in these terms; however, we find it a useful analogy. The two forms bring to mind the picture of the process of making DOPA-melanin, where at the end of the process we find in our beaker a black precipitate as well as a dark supernatant, i.e., a form that goes into solution and a form that precipitates out (higher and lower molecular weight forms).

The above model is further reinforced by the measurements we have obtained from very dark individuals. In the cases of Africans or very dark Indians we find that the straight line absorption dominates the absorption spectrum and the exponential deviation is very difficult to discern. It is well established [8] that in these individuals melanin exists in thoroughly melanized melanosomes, whereas in light skinned individuals we find a distinct deviation from the straight line at wavelengths less than 500 nm. We find no correlation, however, between the appearance of skin and the magnitude of the exponential coefficients. The shorter wavelength absorption is therefore not easily perceivable by the naked eye, if at all.

We found further evidence that there exist two distinct mechanisms of absorption for human melanin when we considered IPD and DPD (Fig 3). It should be kept in mind that in this figure we compare the pigment in the exposed area with that in an adjacent site that is normally pigmented, i.e., we are looking only at the increase in pigment (normal skin compared with another area of normal skin would be a horizontal line on this Fig). We found that in both IPD and DPD the slope of the straight line, in wavelength, increased after exposure, which implies an increase in the visible pigment level. The exponential deviation, on the other hand, was positive in the case of DPD and negative in the

case of IPD. This shows that we are indeed dealing with two distinct mechanisms, as we have found two biologic expressions: in one, the exponential mechanism of absorption increases and in another, it decreases, whereas the long wavelength mechanism increases in both cases. This provides clear biologic evidence that they are acting independently of each other.

Interpreting the observations with IPD and DPD with the two forms of melanin, we could say that in DPD the melanocytes are producing more of both low and high molecular weight melanin (neomelanogenesis). This is evidenced by the fact that the curve for DPD looks very much like the absorption curve for human melanin. On the other hand, in IPD high molecular weight melanin is produced at the expense of the low molecular weight component. Our results indicate that IPD would provide minimal if any photoprotection, which was recently substantiated in the literature [9,10].

It should be further kept in mind that, since the IPD reaction has different spectroscopic characteristics from the DPD reaction, diffuse reflectance spectroscopy can be used to differentiate between these two types of hyperpigmentation. This distinction becomes possible when the hyperpigmentation is clearly evident, with well defined boundaries.

In experiments that are currently in progress in which we measure the diffuse reflectance of skin as a function of tape stripping, we find that for up to 35 strippings, the higher molecular weight form is been removed, as indicated by changes in the slope of the straight line fit, whereas the lower molecular weight form remains intact (as we find no changes in the exponential fit). What we are removing when we first tape strip is stratum corneum that is loaded with melanin "dust" [11]. As we continue stripping, we are probably also removing keratinocytes with melanosomes in them. In our model we make no distinction between melanin dust and melanin in melanosomes; we assume that in both cases it will be in the high molecular weight form since it is insoluble.

In conclusion, (1) we find that human melanin exists in two distinct and independent forms, as far as its absorption properties are concerned. One that is in effect over the entire visible range (straight line in wavelength) and another that becomes apparent at wavelengths shorter than 500 nm. (2) These forms can be correlated with a high molecular weight (insoluble) and a low molecular weight (soluble) component. (3) This model is of biologic significance, as it provides a reasonable interpretation of the spectroscopic information obtained from IPD and from DPD. (4) Clearly visible IPD and DPD reactions have different spectroscopic signatures and therefore can be identified.

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