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A Study of the Oxidation-Reduction State of Synthetic 3,4-Dihydroxy-DL-phenylalanine Melanin

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SUMMARY

This study of the oxidation and reduction of synthetic 3,4-dihydroxy-DL-phenylalanine melanin has demonstrated that (a) the electron exchange with melanin particles is biphasic; (b) about 25% of the indole units are on the surface of melanin particles; (c) melanin exists predominantly in the quinonoid oxidized form, which is suitable for those interactions involved in the formation of charge transfer complexes; (d) the reduced form of melanin is slowly reoxidized by air.

INTRODUCTION

Since the macromolecular pigment melanin can exist in either an oxidized quinonoid or a reduced hydroquinoid form, it belongs to a family of oxidation-reduction polymers in the sense of H. G. Cassidy's definition (1).

However, melanin differs from many synthetic oxidation-reduction polymers in that its π-electron system can be conjugated throughout its structure, and thus the pigment possesses other specific characteristics. As a result of these characteristics, (a) melanin can play a role in biological oxidation-reduction processes as an oxidation-reduction buffer. (b) Melanin is assumed to be a semiconductor (2), and thus its electronic state and related characteristics may elicit unique biological properties. (c) Melanin may play an important role in intermolecular interactions. Its quinonoid form functions as a π-acid and may specifically interact with matching π-bases to form charge transfer complexes, which are similar to those formed with small molecules (3). The formation of charge transfer complexes with π-acids frequently occurs with planar molecules carrying a heteroatom, namely, heteroaromatic compounds such as quinolines, and phenothiazines. Indeed, drugs containing these molecular structures are likely to be bound by the oxidized form of melanin in living animals and thus become localized in tissues containing melanin granules.

Since these properties depend on the oxidation state of melanin, we have studied the oxidation and reduction of melanin formed by the action of tyrosinase on 3,4-dihydroxy-DL-phenylalanine (DL-dopa). With this model system we hoped to obtain insight into the oxidation-reduction properties of the quininoid pigment present in natural melanin granules.

METHODS

Preparation of DL-*dopa melanin.* Melanin was prepared by the action of tyrosinase

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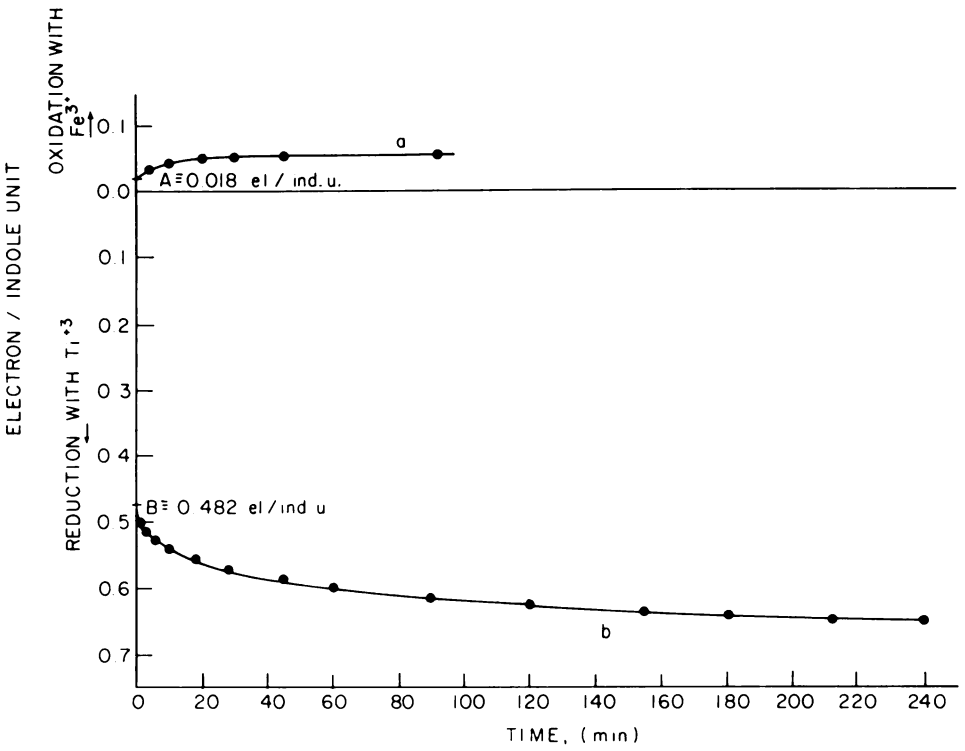

FIG. 1. *Kinetics of oxidation of* DL*-dopa melanin with* Fe^{3+} *(curve a) and its reduction with* Ti^{3+} *(curve b)*

In both experiments the reaction mixture was placed in the polarographic vessel, and the diffusion current of the particular ion was determined polarographically at given intervals. The reaction temperature was 27°. The consumption of the oxidation or reduction reagent is expressed as the number of electrons consumed per indole unit (el./ind. u.). The value 0.0 at the electron per indole unit axis corresponds to the original oxidation-reduction state of the DL-dopa melanin used in the experiment. Points *A* and *B* represent the consumption of the oxidizing or reducing agent at zero time and were obtained by the extrapolation of the corresponding curves, The composition of the reaction mixture in the oxidation experiment (curve *a*) was 160 ml of a 0.25 M sodium oxalate solution, 10 ml of a 2.137×10^{-2} M $Fe_2(SO_4)_3$ solution in 0.5 M H_2SO_4, and 10 ml of a melanin suspension in distilled water containing 32.4 mg of dry melanin, equivalent to 2.219×10^{-4} M indole units. In the reduction experiment (curve *b*), the reaction mixture contained 20 ml of 0.1 M sodium oxalate and 1 M H_2SO_4 solution, 7 ml of a melanin suspension in distilled water containing 34.75 mg of dry melanin, equivalent to 2.38×10^{-4} M indole units, and 5 ml of 7.58×10^{-2} M $TiCl_3$ solution in 1.8 M H_2SO_4.

on dopa according to the procedure of Potts (4). The separation of melanin by centrifugation was accelerated after the fine particles were precipitated by addition of acetic acid; 60 ml of glacial acetic acid were added to 600 ml of reaction mixture. Attempts to separate particles of uniform size were not successful. The material was purified by repeated suspension in distilled water alternating with centrifugation and was finally suspended and stored in water at 3°. Before use, the preparation was resuspended by vigorous stirring for about 5 min, and aliquots were removed. The dry weight of melanin in one of the aliquots was then determined.

Melanin isolated from the suspension by centrifugation and dried in a vacuum had different characteristics from those of the original sample. The particles were larger, more compact, and hard; even after prolonged treatment in water they never regained their original characteristics.

Oxidation-reduction experiments. Fe^{3+} was used as the oxidizing agent, and Ti^{3+} as the reducing agent, in reaction media that were

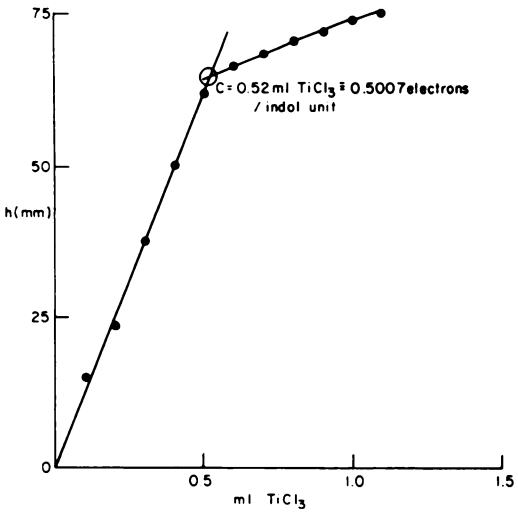

FIG. 2. *Polarographic (amperometric) titration of* DL-*dopa melanin with* Ti^{3+}

The reagent was 0.1042 M $TiCl_3$ solution in dilute sulfuric acid (1:10 by volume). The reaction mixture consisted of 25 ml of 0.1 M oxalic acid and 1.0 M sulfuric acid, and 5 ml of melanin suspension in distilled water containing 15.8 mg of dry melanin, equivalent to 1.082×10^{-4} M indole units. The height (in millimeters) of the diffusion current at −0.9 V (vs. a 0.1 M mercurosulfate electrode) was read immediately after addition of Ti^{3+}. Point *C* was obtained graphically.

suitable for polarographic analysis and simultaneously provided the stabilization of the ions (see legends to figures). The oxidation-reduction potentials of both reagents are appropriate to accomplish these processes. Standard polarographic procedures were used to determine the concentration changes of the ions formed (Fe^{2+}, Ti^{4+}) upon reaction of the reagent (Fe^{3+}, Ti^{3+}) with melanin (5). The polarograph used in the experiments was the Sargent XIX model. The polarographic cell was provided with a mercury dropping electrode (t = 7 min) and with a normal mercurosulfate (1 N Na_2SO_4 and 0.1 N $HgSO_4$) reference electrode separated with an agar bridge. The height of the anodic Fe^{2+} oxidation wave was read at −0.73 V against a 1.0 N $HgSO_4$ electrode; the height of the Ti^{4+} cathodic wave was read at −0.90 V against a 1.0 N $HgSO_4$ electrode. Under the experimental conditions used, 1 cm of the scale (h) corresponded to 2.137×10^{-5} electron equivalent for the oxidation experiment and to 0.9975×10^{-5} electron equivalent for the reduction experiment.

RESULTS AND DISCUSSION

Conjugation of the melanin electron system occurs only when the individual indole rings are in a molecular plane or when the angle of twist of two indole units is small. This conformation of the molecule most probably occurs when melanin is in its highly associated state; e.g., when it is dry or when it is changed from its original highly solvated, dispersed state by precipitation with acids. The black color of these pigments probably results from this large, extended molecular π-electron system. Thus a large number of electronic transitions may

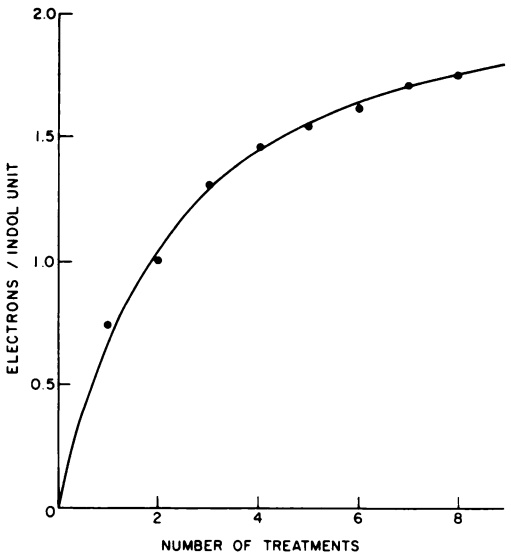

FIG. 3. *Kinetics of slow oxidation of* DL-*dopa melanin by* Fe^{3+}

The sample of dry melanin used in this experiment, 14.6 mg, corresponding to 1.0×10^{-4} M indole units, was suspended in 5 ml of 2.38×10^{-2} M $Fe_2(SO_4)_3$ solution in 0.5 M sulfuric acid (except for the first run, in which 10 ml were used). The reaction mixture was shaken for 48 hr at 27° anaerobically. The consumption of the reagent in the supernatant fluid was determined polarographically. The reagent was then replaced and the experiment was continued. The oxidation process is expressed in numbers of electrons per indole unit.

occur with small differences in energy. Since these transitions are observed in the region of visible light, the color of the pigment appears to be black. Moreover, during oxidation or reduction of melanin, the electronic transfers are presumably not localized on any specific indole unit. Instead, they are reflected by changes in the entire π-electron framework of the molecule.

Nevertheless, the oxidation-reduction processes in quinonoid-hydroquinonoid systems of this macromolecule are based on the same principles as those of monomeric quinones. In general both types of systems are oxidized and reduced as follows:

$$\text{Quinone} + 2e^- + 2\ H^+ \rightleftarrows \text{hydroquinone}$$

Polarographic studies of the monomeric system have been carried out in solution (6). The slope of the polarographic curve and other characteristics prove that both electron and proton transfer are fast processes, and therefore the quinone-hydroquinone system may be directly titrated (7). The collision of reagent or solvent molecules with molecules of insoluble melanin should also result in a fast electron and proton transfer.

As shown in Fig. 1, the kinetics of both the oxidation and reduction of melanin by excess amounts of the reagents indicates two distinct processes, one very fast and the other slow; i.e., one portion of the reagent is consumed instantaneously and the other portion is consumed at a measurable rate. Since the diffusion of the reagent molecules from the bulk of the reaction mixture to the surface of the melanin particles in a well-mixed system should be fast enough to make the entire process instantaneous, the rate-determining process for the slow electron exchange is probably the diffusion of the reagent into the matrix of the particle. Thus, the fast electron exchange probably occurs on the surface of the particles, and the slow process inside the particle. This picture is in good agreement with results of similar oxidation-reduction studies undertaken with synthetic oxidation-reduction polymers such as hydroquinone oxidation-reduction polymers (8). Extrapolation of the kinetic curves to zero time yields values corresponding to the number of exchanged electrons per indole unit of the macromolecule (points *A* and *B*). The value is 0.02 electron/indole unit for the oxidation process and 0.48 electron/indole unit for the reduction process. These values clearly show that melanin exists mainly in the oxidized form and that the ratio between the number of indole units in quinonoid and hydroquinonoid form is 25:1.

The sum of the oxidation and reduction processes gives the total capacity of the melanin in the fast electron processes. From

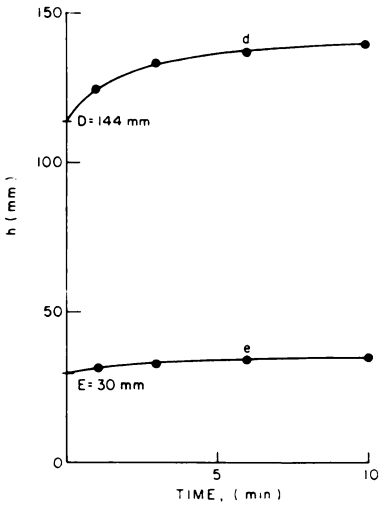

FIG. 4. *Reoxidation of reduced form of* DL-*dopa melanin by air*

A 10-ml aliquot of melanin suspension in distilled water, corresponding to 37.86 μg of pigment and equivalent to 2.59×10^{-4} M indole units, was reduced in 20 ml of 0.1 M sodium oxalate and 1.0 M sulfuric acid solution with 5 ml of 7.64×10^{-2} M $TiCl_3$ in dilute sulfuric acid (1:10 by volume). The concentration changes of the reagent were determined polarographically (curve *d*). The reaction mixture was then incubated for 120 min, and the reduced melanin was separated by centrifugation, washed with dilute sulfuric acid and water under N_2, and suspended in distilled water. Air was bubbled into this suspension for 30 min. Melanin was isolated by centrifugation, and the reduction step was repeated (curve *e*). Points *D* and *E*, obtained graphically by extrapolation, correspond to the consumption of the reagent at zero time. The ratio of the values of *D* and *E* (30:144) indicates that only about 20% of the surface indole was reoxidized by air in 30 min.

the data shown in Fig. 1, this value is 0.5. In addition, the number of electrons required to reduce the quinonoid form of the indole units on the surface of the melanin particles was estimated by adding increasing amounts of Ti^{3+}. As shown in Fig. 2, the titration curve is biphasic, the initial part of the curve being linear and the second part being curved. Intersection of these two phases gave a value of 0.5 electron/indole unit, which closely approximates the value of 0.48 electron/indole unit obtained in Fig. 1. Since 2 electrons are required to reduce the quinonoid form of the indole unit, these findings indicate that only about 25% of the total number of indole units in the melanin particles are readily accessible to these processes.

The graph in Fig. 3 represents the slow oxidation process of melanin with Fe^{3+}. The curve approaches an asymptote of 2 electrons/indole unit. Since melanin exists predominantly in the quinonoid oxidized form, this extremely slow oxidation of melanin by Fe^{3+} is not related to the oxidation-reduction processes within the quinone-hydroquinone oxidation-reduction system, but may represent cross-linking. The removal of 2 electrons/indole unit suggests a dehydrogenation process in which 2 hydrogen atoms are removed. Since the formation of a new σ bond between two neighboring indole units requires the removal of 1 electron for each indole unit, the data suggest the formation of two σ bonds per indole unit.

Figure 4 shows the results of an experiment in which the melanin sample was first partially reduced by treatment with excess Ti^{3+} for 2 hr and then reoxidized by bubbling air into the suspension for approximately 30 min. Only about 20% of the partially reduced indole units on the surface of the melanin were converted to their original state, which suggests that the reoxidation of the reduced form of melanin is rather slow.

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