Studies Related to the Chemistry of Melanins. Part 15.[1] The Electron Transfer and Free Radical Properties of Dopa-melanin

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The e.s.r. signal of autoxidative dopa-melanin remained unchanged when the melanin was treated with the following reagents: potassium nitrosodisulphonate, *NNN'N'*-tetramethyl-*p*-phenylenediamine bromide, β-NADH, chlorpromazine, ascorbic acid, sodium dithionite, potassium hexacyanoferrate(III), or cerium(IV), copper(II), or zinc ions, or water, showing that the radical sites are inaccessible to chemical attack. The interaction of melanin's free radicals with copper(II) ions is not chemical in nature; the number of bound copper(II) ions was determined.

ELECTRON spin resonance signals from natural melanins were first detected by Commoner, Townsend, and Pake,[2] who attributed these to free radicals trapped in the polymer. The trapping of free radicals by a growing polymer has been observed with methacrylate polymers.[3] The former authors and also Mason, Ingram, and Allen [4] suggested that melanin might act as a ' free radical trap ', protecting living cells against toxic free radicals produced, for example, by solar irradiation. Little is known about such reactions although it has been shown that diphenylpicrylhydrazyl reacts with melanin not *via* a radical–radical reaction but by hydrogen abstraction to form diphenylpicrylhydrazine.[5]

The e.s.r. spectrum of a melanin generally consists of a single, usually structureless absorption line, with a *g* value near 2, and a paramagnetism that is permanent.[6] The free radicals are very stable; thus the signal is not eliminated when melanin is heated in oxygen at 200—500 °C, nor when it is boiled with acid or alkali. This stability was attributed to the radicals being buried deeply in the three-dimensional polymeric structure. The signal was however destroyed when the melanin was treated with a sufficiently high concentration of copper(II) ions, and it was suggested that these small ions were able to penetrate to the deeply buried free radicals. However the question as to why copper(II) ions but not oxygen molecules penetrate to the radical sites was unanswered.

Conflicting results were reported for the effect of ascorbic acid on the e.s.r. signal of melanins. Blois, Zahlan, and Maling [6] showed that the e.s.r. signal of squid-melanin was unaffected by mild treatment with an excess of ascorbic acid. In contrast, Mason, Ingram, and Allen [4] reported that the e.s.r. absorption of *Sepia*-melanin was halved in intensity, whereas Chauffe, Windle, and Friedman [7] found a 64% decrease in the e.s.r. signal intensity when wool-melanin was heated under reflux with 2M-ascorbic acid. In these cases natural melanins were used, which may have contained attached protein together with paramagnetic ions [copper(II), iron(III), or manganese(II)]. Traces of copper(II) ions catalyse the oxidation of ascorbic acid below pH 7.6,[8] and ascorbic acid is rapidly decomposed when heated.

Van Woert [9] reported that melanin catalysed the *in vitro* oxidation of reduced nicotinamide-adenine dinucleotide (β-NADH) and *p*-phenylenediamine, and suggested that this was related to melanin's free radical property. The phenothiazines inhibit the ability of melanin to catalyse the oxidation of β-NADH, and Van Woert attributed this to a reduction in the number of

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[7] L. Chauffe, J. J. Windle, and M. Friedman, *Biophys. J.*, 1975, **15**, 565.
[8] H. Mohler and H. Lohr, *Helv. Chim. Acta*, 1938, **21**, 485.
[9] M. H. Van Woert, *Life Sciences*, 1967, **6**, 2605; *Proc. Soc. Exp. Biol. Med.*, 1968, **129**, 165.

melanin free radicals. Although this explanation appears to be consistent with Forrest, Gutmann, and Keyser's report [10] that chlorpromazine reduces the e.s.r. signal of melanin by *ca.* 50% it is nevertheless very improbable because of the high stability of melanin radicals. A possible alternative explanation to a radical mechanism is that oxidation of β-NADH occurs *via* an electron-transfer process. Quinone units in the melanin might act as electron acceptors, subsequent reoxidation of the reduced melanin being achieved by molecular oxygen. As phenothiazines are known to bind to melanin,[11] a more likely reason for the observed inhibition is that bound phenothiazine molecules block the quinonoid reaction centres of the melanin polymer.

Because of the interest of melanin as a possible radical trap, we carried out experiments to determine whether free radicals, such as potassium nitrosodisulphonate or *NNN'N'*-tetramethyl-*p*-phenylenediamine bromide, or oxidising or reducing agents could react with melanin. In view of the conflicting results reported on the effect of ascorbic acid on the e.s.r. signal of natural melanins we decided to repeat these experiments using synthetic melanin, which was free from protein and paramagnetic ions. As even melanins prepared enzymically *in vitro* may contain protein and copper(II) ions, all melanin samples used in the present work were of autoxidative dopa-melanin. We also attempted to show that the stable free radical property of melanin was not concerned in the ability of melanin to catalyse the *in vitro* oxidation of β-NADH, thus favouring the electron-transfer mechanism. In addition we investigated the possible catalytic effect of melanin on its own formation from dopa by autoxidation.

It seemed that reduction in the e.s.r. signal intensity reported by some previous workers on treatment of melanin with ascorbic acid or chlorpromazine may have arisen because the spectra were measured on equal weights of treated and untreated melanin; if ascorbic acid or chlorpromazine remained held to the polymer the weight of the treated sample would represent a smaller weight of pigment than that of the untreated sample.

Melanins are hygroscopic and can bind up to 30% of their own weight of water.[6] The e.s.r. signal intensity of squid-melanin was found to decrease with increase in hydration of the sample, and Blois *et al.* suggested two possible explanations. The first, that the small water molecules are able to penetrate to the deeply buried free radical sites, seemed unlikely as it was shown [6] that oxygen molecules are unable to penetrate. The alternative, that the decrease in signal intensity might be due simply to a variation in spin-lattice relaxation time, could not be verified as the e.s.r. signals showed saturation effects over the range of incident microwave power used. In contrast, our spectra showed no saturation effects. A sample of melanin containing 17% by weight of water was prepared by partly drying a melanin slurry for 20 h *in vacuo* over calcium chloride. The e.s.r. signal intensity of the hydrated sample was compared with that of a control sample that was dried to constant weight *in vacuo* over phosphoric oxide. The signal intensities were identical, confirming that the melanin free radicals are not destroyed by chemical reaction with bound water molecules.

When melanin was treated either with potassium nitrosodisulphonate at pH 9.0 or with *NNN'N'*-tetramethyl-*p*-phenylenediamine bromide in acidic solution, the e.s.r. signal amplitude remained identical with that of a control, showing that no radical–radical reaction had occurred.

Treatment of melanin with an excess of ascorbic acid at pH 9.0 resulted in no change in e.s.r. signal intensity. Rothman [12] reported that melanin obtained from the ink sac of the squid or produced by the autoxidation of dopa was lightened in colour from jet black to light tan by ascorbic acid; but we observed no change in colour in the case of autoxidative dopa-melanin.

Treatment of melanins with diazomethane [13] results in the methylation of carboxy- and phenolic groups to give methoxy-groups, which can be determined by the Zeisel method. Reduction of indole-5,6-quinone units in the melanin polymer by ascorbic acid would result in an increase in the number of 5,6-dihydroxyindole units and an increase in methoxy-value after exhaustive methylation with diazomethane. The methoxy-value (19.3%) of the ascorbic acid-treated sample differed only slightly from that of a control (18.0%) that was not so treated. This showed that the melanin had not been reduced to a significant extent. Ascorbic acid is a weaker acid (K_1 2×10^{-5}, K_2 6×10^{-13}) than dehydroascorbic acid (K_1 6×10^{-4}; K_2 1.2×10^{-8}) and oxidation proceeds with a decrease in pH. The rate of decrease in pH was not affected by the presence or absence of melanin, showing that melanin does not catalyse the autoxidation of ascorbic acid.

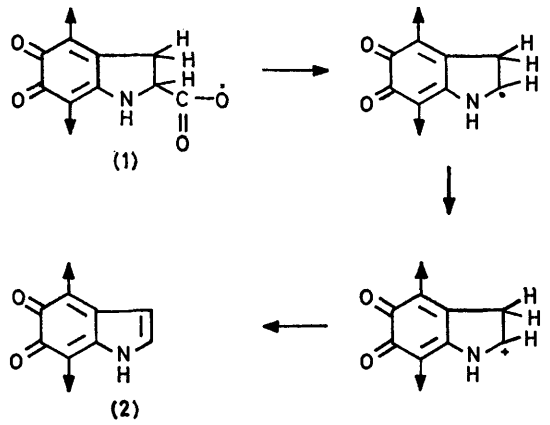
Both the colour and the e.s.r. signal intensity of melanin were unchanged after treatment with sodium dithionite solution at pH 11.0. The sample was exhaustively methylated with diazomethane and methoxy-values were determined as described above. The value found (18.2%; *cf.* 18.0% for a control sample) showed that no overall increase in the number of 5,6-dihydroxyindole units had taken place. The melanin was appreciably lightened in colour when the reduction with sodium dithionite was carried out under nitrogen in strongly alkaline solution. Rapid aerial oxidation of the reduced melanin occurred, the colour of the sample darkening to jet black after removal of the solvent and drying. Similarly, Das *et al.*[14] reported that mela-

[10] I. S. Forrest, F. Gutmann, and H. Keyzer, *Rev. Agressol.*, 1966, **7**, 147.
[11] A. M. Potts, *Invest. Ophthalmol.*, 1962, **1**, 522 and 1964, **3**, 399; M. S. Blois in 'Advances in Biology of Skin,' eds. W. Montagna, R. B. Stoughton, and E. J. Van Scott, vol. XII, Appleton-Century-Crofts, New York, 1972, p. 65.
[12] S. J. Rothman, *J. Invest. Dermatol.*, 1942, **5**, 67.
[13] G. A. Swan and A. Waggott, *J. Chem. Soc.* (*C*), 1970, 1409.
[14] K. C. Das, M. B. Abramson, and R. Katzman, *J. Neurochem.*, 1976, **26**, 695.

nins which had been reduced by sodium borohydride were unstable at room temperature and darkened substantially on drying.

If indeed the melanin radicals are deeply buried, it is surprising that treatment with an excess of copper(II) ions destroys the e.s.r. signal. A possible explanation is that the buried radicals are oxidised by an electron-transfer process, and to test this the effects of other oxidising agents were investigated. Melanins are rapidly oxidised [15] by potassium hexacyanoferrate(III). The e.s.r. signal intensity of melanin was however unchanged by potassium hexacyanoferrate(III) solution at pH 10.0. Similarly, the e.s.r. signal was not destroyed after the melanin had been treated with an acidic solution of cerium(IV) sulphate. Treatment with 3.6N-sulphuric acid at 60—70 °C removed cerium(III) ions bound to the oxidised melanin and their concentration (28% by weight) was determined by u.v. spectrometry.

The above results suggested that oxidation of the melanin radicals by copper(II) ions was unlikely. An alternative explanation that copper(II) ions catalyse decarboxylation reactions, *e.g.* (1) ⟶ (2) as shown in the Scheme, was ruled out as the e.s.r. signal of melanin



SCHEME

was not eliminated when the sample was decarboxylated (230 °C; 0.1 mmHg; 12 h). Zinc salts catalyse [16] the rearrangement of aminochromes to 5,6-dihydroxyindoles and by analogy might catalyse the reaction (1) ⟶ (2). Studies by Sarna and Lukiewicz [17] on the effect of zinc ions on the e.s.r. signal amplitude of melanin pigment from damaged amphibian eggs and embryos were difficult to interpret, different melanin preparations giving widely different responses. Natural melanins contain paramagnetic ions that partly quench the true melanin free radical resonance. Replacement of these ions by diamagnetic zinc ions results in an increase in e.s.r. signal intensity.

Autoxidative dopa-melanin contains no paramagnetic ions and treatment of this melanin with zinc sulphate solution showed the true effect on the radicals. The e.s.r. signal intensity was unchanged, indicating that the diamagnetic zinc ions did not react with the radicals.

The above results are consistent with the work of Sarna, Hyde, and Swartz,[18] who showed that the apparent reaction of melanin free radicals with paramagnetic ions, *e.g.* copper(II) ions, was a purely physical consequence of magnetic dipolar interaction. Paramagnetic lanthanoid ions, *e.g.* gadolinium(III), eliminated the e.s.r. signal of bovine-choroid melanin, while chemically similar diamagnetic ions, *e.g.* lanthanum(III), were without effect. The melanin samples studied were isolated by a mild extraction procedure that left protein still incorporated in the granules, and the results are therefore subject to two limitations. First the true melanin e.s.r. signal might be partly quenched by paramagnetic ions accompanying the non-hydrolysed peptide remnants, and secondly metallic ions might preferentially bind to protein, which would lead to erroneous conclusions about the true melanin binding sites. Autoxidative dopa-melanin contains neither admixed protein nor paramagnetic ions and is not subject to these limitations.

Melanin was treated with an excess of copper(II) ions and the amount of bound copper(II) ions was determined. The melanin was boiled with concentrated nitric acid and copper(II) ions were extracted as their diethyldithiocarbamate [19] complex which was determined colorimetrically. It was found that 90 mg of melanin bound 0.75 mg of copper(II) ions after treatment with an excess of copper(II) ions (12.8 mg). If one assumes a monomer molecular weight of 147, this is equivalent to one bound copper(II) ion for every 50 units of monomer. Sarna *et al.*[18] reported 6×10^{20} metal binding sites per g of dried bovine choroid-melanin. This value, equivalent to 1 bound metal ion for every 7 monomers is higher than that for autoxidative dopa-melanin. A possible explanation is that some metal ions bind to protein in the natural melanin.

The e.s.r. signal was not restored when melanin was washed four times with N-hydrochloric acid after treatment with copper(II) ions. The acid-washed melanin (70 mg) retained 0.5 mg of copper(II) ions, showing that they were not easily removed. In contrast it was found [18] that the intensity of the e.s.r. signal of bovine choroid-melanin which had been treated with copper(II) ions increased on lowering the pH. The number of bound copper(II) ions decreased, indicating that the ions were more easily removed from the natural melanin. This might be attributed to binding of the metal ions to protein sites.

Evidence against the free-radical explanation of the ability of melanins to catalyse the oxidation of β-NADH was that the e.s.r. signal intensity of melanin was un-

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[16] P. Fischer and G. Derouaux, *Compt. rend. Soc. Biol.*, 1950, **144**, 707.

[17] T. Sarna and S. Lukiewicz, *Folia Histochemica et Cytochemica*, 1972, **10**, 265.

[18] T. Sarna, J. S. Hyde, and H. M. Swartz, *Science*, 1976, **192**, 1132.

[19] A. I. Vogel, 'A Textbook of Quantitative Inorganic Analysis,' Longman, London, p. 666.

changed after the melanin had been treated with β-NADH at pH 8.0. The catalytic abilities of two samples of melanin, only one of which had been treated with an excess of copper(II) ions (and then showed no e.s.r. signal), were compared. The rate of oxidation of β-NADH in the presence of either sample, followed manometrically in the Warburg apparatus [20] at 37° and pH 8.0, was the same, showing that bound copper(II) ions do not catalyse the oxidation of the reduced melanin.

Melanin was treated with chlorpromazine at pH 5.0 and the bound chlorpromazine was determined by extracting the melanin with ethanol and measuring its concentration in the resulting extract spectrophotometrically. The melanin was found to bind its own weight of chlorpromazine. The e.s.r. signal amplitude of the chlorpromazine-containing sample was identical with that of a control sample (on a melanin weight basis), showing that treatment with chlorpromazine resulted in no reduction in the number of melanin radicals.

To determine whether melanin might catalyse its own formation, the rate of autoxidation of dopa in the presence or absence of melanin was measured at pH 6.8 in both 0.1M-phosphate and 0.50M-citrate buffer in the Warburg apparatus. The autoxidation proceeded very slowly after a long induction period and no catalysis by melanin was detected.

We conclude that the free radicals of melanin are inaccessible to attack by chemical reagents such as metallic ions, stable free radicals, β-NADH, chlorpromazine, and oxidising and reducing agents. Reduced melanin is very readily oxidised back to the quinone by air.

EXPERIMENTAL

(±)-3,4-Dihydroxyphenylalanine was obtained from B.D.H. Chemicals Ltd. and was chromatographically pure. Except where otherwise stated melanins were dried *in vacuo* over phosphoric oxide for 3 days.

Electron Spin Resonance Spectra.—These were recorded on dry, powdered melanin samples (35 mg) at room temperature using a Hilger and Watts Microspin X-Band bridge spectrometer with a rectangular cavity operating at 9 400 MHz in the H_{012} mode. Magnetic field modulation at 100 kHz with phase sensitive detection was employed and first derivative curves were plotted. *g* Values were very close to that of diphenylpicrylhydrazyl and the microwave power range employed was such that saturation effects were not observed.

Preparation of samples. Oxygen was passed for 18 h at 37 °C through a solution of (±)-3,4-dihydroxyphenylalanine (250 mg) in 0.1M-phosphate buffer at pH 8.0 (100 ml). Samples (50 ml) were taken, hydrochloric acid (2N; 5 ml) was added, and the melanin was centrifuged and washed with water (10 ml). The resulting slurry (containing *ca.* 60 mg) was used in the experiments below. In all experiments a control sample (50 ml) was taken and the melanin isolated as above. This sample was used as a standard for comparison of e.s.r. signal intensities after both samples had been washed and dried under identical conditions. When 'identical signal amplitude' is stated the observed peak heights were equal to within ±5%.

Treatment of Melanin with Potassium Nitrosodisulphonate.—Potassium nitrosodisulphonate was prepared as described by Singh.[21] To a slurry of the melanin (*ca.* 60 mg) in distilled water (10 ml) was added a solution of the radical (200 mg) in sodium hydroxide solution (pH 9.0; 10 ml). The suspension was stirred at room temperature for 2 h during which time the pH was maintained close to 9.0 by dropwise addition of 0.05N-sodium hydroxide. Hydrochloric acid (2N; 6 ml) was added to decompose the excess of potassium nitrosodisulphonate,[22] and the melanin was collected, washed with hydrochloric acid (0.3N; 4 × 20 ml), and dried.

Treatment of Melanin with NNN'N'-*Tetramethyl*-p-*phenylenediamine Bromide.*—The radical was prepared as described by Wurster.[23] To a melanin slurry (*ca.* 60 mg) was added a solution of the radical (50 mg) in aqueous acetic acid (50% v/v; 20 ml) and the suspension was stirred at room temperature for 5 min. The melanin was precipitated by addition of hydrochloric acid (2N; 10 ml), removed by centrifugation, washed with hydrochloric acid (0.1N; 2 × 5 ml), and dried.

Treatment of Melanin with Ascorbic Acid.—(*a*) To a melanin slurry (*ca.* 60 mg) in distilled water (10 ml) was added ascorbic acid (200 mg) in distilled water (30 ml). The pH was adjusted to 9.0 and maintained close to this value by dropwise addition of 0.05N-sodium hydroxide while the mixture was stirred at room temperature for 21 h. Hydrochloric acid (2N; 5 ml) was added, and the melanin was collected, washed with hydrochloric acid (0.1N; 4 × 10 ml), and dried.

(*b*) In a second experiment the reaction mixture was stirred for 5 days.

(*c*) A solution of ascorbic acid (200 mg) in sodium hydroxide solution (20%; 10 ml) was added under nitrogen to a melanin slurry (*ca.* 60 mg) in sodium hydroxide solution (20%; 10 ml) and the suspension was stirred under nitrogen at room temperature for 5 h. The melanin was precipitated by addition of hydrochloric acid (4N; 30 ml), removed by centrifugation, washed with hydrochloric acid (0.1N; 4 × 10 ml), and dried.

Oxidation of Ascorbic Acid.—(*a*) The pH of a solution of ascorbic acid (200 mg) in distilled water (20 ml) was adjusted to 9.0 by dropwise addition of 0.5N-sodium hydroxide. The solution was stirred at room temperature for 90 min and pH readings were taken at 5 min intervals.

(*b*) Experiment (*a*) was repeated using a mixture of ascorbic acid (200 mg) and melanin (*ca.* 60 mg) suspended in distilled water (20 ml).

Treatment of Melanin with Sodium Dithionite.—(*a*) To a melanin slurry (*ca.* 60 mg) was added a solution of sodium dithionite (200 mg) in distilled water (20 ml). The pH was adjusted to 11.0 and maintained close to this value by dropwise addition of 0.05N-sodium hydroxide while the mixture was stirred at room temperature for 3 h. The mixture was acidified with hydrochloric acid (2N; 5 ml), and the melanin was collected, washed with hydrochloric acid (0.1N; 3 × 15 ml), and dried.

(*b*) A solution of sodium dithionite (200 mg) in sodium hydroxide solution (20%; 10 ml) was added under nitrogen to a melanin slurry (*ca.* 60 mg) in sodium hydroxide solution

[20] M. Dixon, 'Manometric Methods,' Cambridge University Press, 1943.
[21] R. P. Singh, *Canad. J. Chem.*, 1966, **44**, 1994.
[22] J. H. Murib and D. M. Ritter, *J. Amer. Chem. Soc.*, 1952, **74**, 3394.
[23] C. Wurster, *Ber.*, 1879, **12**, 1807.

(20%; 10 ml). The mixture was stirred under nitrogen for 5 h at room temperature. Hydrochloric acid (4N; 30 ml) was added under nitrogen and the light brown melanin was precipitated.

Treatment of Melanin with Potassium Hexacyanoferrate(III).—A solution of potassium hexacyanoferrate(III) (500 mg) in distilled water (20 ml) was added to a melanin slurry (*ca.* 60 mg). The pH was adjusted to 10.0 and maintained close to this value by dropwise addition of 0.05N-sodium hydroxide while the mixture was stirred at room temperature for 20 min. Hydrochloric acid (2N; 10 ml) was added, and the melanin was collected, washed with hydrochloric acid (0.05N; 5 × 20 ml), and dried.

Treatment of Melanin with Cerium(IV) *Sulphate.*—A solution of cerium(IV) sulphate (120 mg) in sulphuric acid (1N; 10 ml) was added to a melanin slurry (*ca.* 60 mg) and the suspension was stirred at room temperature for 5 min. The melanin was removed by centrifugation, washed with sulphuric acid (0.05N; 2 × 10 ml), and dried. The dry melanin (50 mg) was stirred for 30 min at 60—70 °C with sulphuric acid (3.6N; 10 ml); water (35 ml) was added and the mixture was centrifuged. The acidic supernatant was decanted and its u.v. spectrum was recorded. Absorption at $\lambda_{max.}$ 254 nm (ε 770) showed the presence of cerium(III) ions (14 mg).

Treatment of Melanin with Zinc Sulphate.—A solution of zinc sulphate (250 mg) in distilled water (10 ml) was added to a melanin slurry (*ca.* 60 mg) and the mixture was stirred at room temperature for 5 min. The melanin was removed by centrifugation, washed with distilled water (2 × 20 ml), and dried.

Treatment of Melanin with Copper(II) *Sulphate.*—(*a*) A solution of copper(II) sulphate pentahydrate (250 mg) in distilled water (10 ml) was added to a melanin slurry (*ca.* 60 mg) and the mixture was stirred at room temperature for 5 min. The melanin was removed by centrifugation, washed with distilled water (10 ml), and dried.

(*b*) Experiment (*a*) was repeated but the melanin was not dried after treatment with copper(II) sulphate. Concentrated nitric acid (20 ml) was added to the melanin and the mixture was boiled for 3 h. The solution was evaporated to dryness, the residue was dissolved in sulphuric acid (0.1N; 10 ml), and sodium diethyldithiocarbamate solution (0.1%; 10 ml) was added. The copper(II) diethyldithiocarbamate complex was extracted with carbon tetrachloride (6 × 5 ml) and determined colorimetrically against standard copper(II) solutions. The dry weight of melanin was determined by drying to constant weight an identical melanin sample not treated with copper(II) sulphate. The melanin (dry weight 90 mg) bound copper(II) ions (0.75 mg).

(*c*) Experiments (*a*) and (*b*) were repeated but the melanin was washed with hydrochloric acid (1N; 4 × 10 ml) after treatment with copper(II) sulphate. The melanin (70 mg) bound copper(II) ions (0.5 mg).

Oxidation of Reduced Nicotinamide–Adenine Dinucleotide (β-*NADH*).—The oxidation was studied manometrically in the Warburg [20] apparatus at 37 °C. The main compartment of each flask contained melanin (15 mg) suspended in 0.1M-phosphate buffer of pH 8.0 (4.0 ml) and the side-arm contained β-NADH (10.3 mg) in 0.1M-phosphate buffer of pH 8.0 (0.8 ml). The inner cup contained sodium hydroxide solution (10%; 0.2 ml) and the gas phase was 100% oxygen.

Treatment of Melanin with β-*NADH.*—The pH of a melanin slurry (*ca.* 60 mg) in distilled water (10 ml) was adjusted to 8.0 by dropwise addition of 0.05N-sodium hydroxide. A solution of β-NADH (50 mg) in 0.1M-phosphate buffer of pH 8.0 (10 ml) was added and the solution was stirred at room temperature for 20 h. The melanin was precipitated by addition of hydrochloric acid (2N; 5 ml), removed by centrifugation, washed with hydrochloric acid (0.1N; 5 × 10 ml), and dried.

Treatment of Melanin with Chlorpromazine.—A solution of chlorpromazine hydrochloride (300 mg) in distilled water (30 ml) was adjusted to pH 5.0 by dropwise addition of 10% sodium hydroxide solution. The solution was added to a melanin slurry (*ca.* 60 mg) and the mixture was stirred at room temperature for 20 h. The melanin was precipitated by addition of hydrochloric acid (2N; 5 ml), removed by centrifugation, washed with hydrochloric acid (0.1N; 3 × 10 ml), and dried. The dry melanin was stirred for 2 h with ethanol (50 ml); hydrochloric acid (2N; 2 drops) was added and the suspension was centrifuged. The ethanolic supernatant was decanted and its u.v. spectrum was recorded. Absorption at $\lambda_{max.}$ 257 nm (ε 32 400) showed that the melanin sample contained chlorpromazine (26 mg).

The research reported herein has been sponsored in part by the United States Army through its European Research Office. We are grateful to May and Baker Ltd. for a gift of chlorpromazine hydrochloride and thank Professor D. H. Whiffen and Dr. K. W. Baldry for advice.

[6/2181 *Received*, 26*th November*, 1976]