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Sulphydryl compounds in melanogenesis. Part I. Reaction of cysteine and glutathione with 5,6-dihydroxyindoles.

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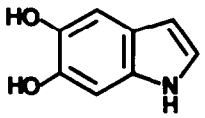
Abstract- Tyrosinase catalyzed oxidation of 5,6-dihydroxyindole (1) in the presence of glutathione leads in the early stages to an unstable 1:1 adduct which has been isolated and identified as 4-S-glutathionyl-5,6-dihydroxyindole (2). Under similar conditions, 5,6-dihydroxy-1-methylindole (3) reacts with L-cysteine ethyl ester (4a) or N-acetyl-L-cysteine (4b) to give the analogous adducts 5a or 5b, presumably by a mechanism involving the addition of the thiol to the enzymically generated 1-methyl-5,6-indolequinone. Such a mechanism is discussed in the light of the observed ability of some one-electron oxidants, such as ammonium persulphate, Fe^{2+}-EDTA/H_2O_2 and copper ions, to effect the coupling of 4a and 4b with 3 to give the same adducts 5a and 5b.

Sulphydryl compounds have long been recognized as one of the controlling factors in the biosynthesis of melanins, the primary pigments found in mammals.[1,2] In a series of papers carried out in the 1940's Rothman and coworkers[3,4] reported that glutathione is able to inhibit the tyrosinase catalyzed oxidation of dopa to melanin. On the basis of this and other observations[5] it was assumed that glutathione as well as other low molecular weight sulphydryl compounds could exert their inhibitory action by combining with the copper ions present at the active site of tyrosinase.

However, as a result of more recent studies, it is now generally agreed that the regulatory role of sulphydryl compounds is not related to the inhibition of tyrosinase, but rather to their ability of scavenging highly reactive quinonoid intermediates formed in the early stages of melanogenesis[1]. Most of the work in this area has been directed to the interaction of glutathione or cysteine with enzymically generated dopaquinone leading to glutathione[6] or cysteine-dopa adducts,[7,8] the latter being the main precursors of reddish-brown pigments known as phaeomelanins[1]. Whether cysteine and related thiol compounds, e.g. glutathione, can also intervene in the later stages of the melanin pathway has so far received sporadic attention. Bouchilloux and Kodja[9] showed that the oxidation of 5,6-dihydroxyindole (1) to melanin in the



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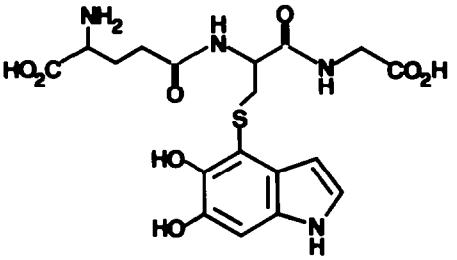

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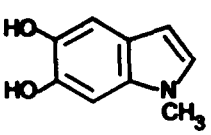
presence of tyrosinase is significantly inhibited by an excess of glutathione. On the basis of paper chromatographic and spectroscopic evidence, these authors suggested that an indole-glutathione adduct was formed in the early stages of the reaction. A similar conclusion was later drawn by Mason and Peterson[10] in the course of a survey on the reactions of aminoacids with 1 and other melanin precursors.

We report now the isolation of the adduct described by Bouchilloux and Kodja as well as other aspects of the reactivity of sulphydryl compounds towards 5,6-dihydroxyindoles.

In preliminary experiments, the tyrosinase-catalyzed oxidation of 1 in the presence of glutathione was monitored spectrophotometrically. It was found that the course of the reaction is markedly dependent not only on the thiol/indole ratio, but especially on the concentration of 1. Thus, for example, with a five-fold excess of glutathione , formation of the colourless adduct is observed only at a concentration of 1 lower than 3.5 x 10-3 M, whereas as the concentration increases, self-condensation of 1 to give melanin becomes prevailing. A procedure was then developed which allowed the isolation of the reported adduct as a glassy oil, homogeneous on TLC and HPLC, $[\alpha]_D^{25°}$ =-23.3°, λ_{max} 295 and 307 nm. The product was formulated as 4-S-glutathionyl-5,6-dihydroxyindole (2) on the basis of the ^{1}H-NMR spectrum showing, in addition to the glutathione resonances, two doublets at δ 6.50 and 7.25 for the H-3 and H-2 protons of the indole ring, respectively, and a singlet at δ 7.06 attributable to the H-7 proton. That coupling with the thiol involved the 4 position of the indole ring was further substantiated by the presence in the ^{13}C-NMR spectrum of three doublets centred at δ 125.75, 100.86 and 100.65 attributable to C-2, C-3 and C-7 carbons, respectively, and a singlet at δ 107.44 due to the C-4 carbon.

When the enzymic oxidation of 1 was carried out in the presence of cysteine, no substantial difference was observed in the spectroscopic course of the reaction with respect to glutathione. Attempts to isolate the cysteine adduct were unsuccessful, and we eventually resolved to characterize the reaction product arising from the tyrosinase catalyzed oxidation of 5,6-dihydroxy-1-methylindole (3) with cysteine ethyl ester 4a. The ethyl acetate extractable adduct thus obtained was isolated in crystalline form as the acetyl derivative and identified as 5a. Likewise, reaction of 3 with N-acetylcysteine (4b) led to the corresponding adduct which was isolated after permethylation with ethereal diazomethane and characterized as 5b.



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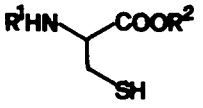
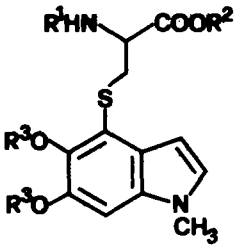


4a: $R^1 = H, R^2 = CH_2CH_3$

4b: $R^1 = COCH_3, R^2 = H$



5a: $R^1 = R^3 = COCH_3$, $R^2 = CH_2CH_3$

5b: $R^1 = COCH_3$, $R^2 = R^3 = CH_3$

Parallel experiments were aimed at ascertaining whether the coupling of sulphydryl compounds with 5,6-dihydroxyindoles could also be brought about by non-enzymic oxidizing systems. When a mixture of 3 with either 4a or 4b was oxidized with potassium ferricyanide, a reagent widely used for the coupling of sulphydryl compounds with o-diphenols,[11,12] no detectable formation of indole-thiol adducts was observed under a variety of conditions. However, satisfactory coupling of the substrates could be obtained when the Fenton reagent (Fe^{2+}-EDTA/H_2O_2)[13] or ammonium persulphate were used as oxidizing agents. In both cases the reaction afforded, besides unchanged material, the corresponding adducts 5a and 5b in about 20% yield. Interestingly, at physiological pHs, e.g. pH 7.5, cupric ions, which are normally present in melanin containing tissues,[14] were also able to mediate the conjugation of 3 with 4b to give 5b, albeit in low yield (about 5%).

Taken together, the results of our study provide evidence that under biomimetic conditions sulphydryl compounds of biological interest, such as glutathione and cysteine, are capable of efficiently reacting with 5,6-dihydroxyindoles to give 1:1 adducts. As far as the mechanism of the conjugation is concerned, it is known that tyrosinase is capable of effecting the two-electron oxidation of catechols to o-quinones,[15] whereas persulphate and Fe^{2+}-EDTA/H_2O_2 system are established one-electron oxidants, the former by electron transfer[16] and the latter through the action of the hydroxyl radical.[17] The fact that, whatever the oxidizing system used, the reaction leads only to the C-4 indole-thiol adducts would suggest that a common mechanism is eventually operative, involving the addition of the sulphydryl group to the highly reactive 5,6-indolequinone.[18] This reaction has a precedent in the synthesis of cysteinyldopas by addition of cysteine to enzymically[7] or chemically[11] generated dopaquinone.

EXPERIMENTAL

M.ps. were determined with a Kofler hot-stage apparatus and are uncorrected. UV spectra were recorded with a Perkin Elmer Mod. 550S spectrophotometer. ^{1}H-NMR (200 MHz) and ^{13}C-NMR (50 MHz) spectra were performed on a Varian XL-200 spectrometer (δ values are referred to tetramethylsilane as internal standard). Electron impact and fast atom bombardment mass spectra

were determined with a Kratos MS 50 mass spectrometer. Besides the molecular ion, the most abundant ions in the mass spectra (above m/e 100) are given with their relative intensities.Analytical and preparative TLC were carried out on a precoated silica gel F-254 plates (0.25 and 0.50 mm layer thickness, E. Merck). Proportion given for mixed solvents are by volume. The chromatograms were examined by UV irradiation at λ 366 and 254 nm. Sephadex G-10 used for column chromatography was purchased by Pharmacia Fine Chemicals (Uppsala, Sweden). Preparative HPLC was carried out on a Waters model 6000A instrument, using a 1x25 cm RP18 Lichrosorb Hibar column (E. Merck). The mobile phase was CH_3CN/H_2O 1:1 and the flow rate was maintained at 4ml/min. Detection was carried out with a UV spectrophotometer Waters model 480 (λ =235 and 330 nm). Mushroom tyrosinase was purchased as a lyophilized powder (4300 units/mg) from Sigma Chemical Co. (St. Louis, MO, USA) and was freed from metal impurities by chromatography on Chelex-100 resin (Na+ form). Distilled water and buffers were passed over Chelex 100 resin before use.5,6-Dihydroxyindole (1)[19] and 5,6-dihydroxy-1-methylindole (3)[20] were prepared as previously reported.

Tyrosinase catalysed conjugation of 5,6-dihydroxyindole with glutathione.

To a solution of 1 (20 mg) and glutathione (200 mg) in 0.025 M phosphate buffer, pH 6.8 (40 ml), a solution of mushroom tyrosinase (23,000 units) in 5 ml of the same buffer was added under vigorous stirring. After 2 h, the pale yellow reaction mixture was slightly acidified to pH 6 by addition of 0.2 M HCl and extracted twice with ethyl acetate to remove traces of unreacted 1. The aqueous layer was evaporated to dryness in vacuo at 30°C and the residue,taken up in water, was chromatographed on a 1.5x100 cm Sephadex G-10 column using water as the eluent. Fractions of 5 ml each were collected and monitored by UV. Fractions 22-25, which showed absorption maxima at 295 and 307 nm, were evaporated to dryness at 30°C under reduced pressure to give 4-S-glutathionyl-5,6-dihydroxyindole (2, 29 mg, 48% yield), λmax (H_2O) 295, 307 nm; $[\alpha]_D^{25°}$=-23.3°(c=1.8, H_2O); FAB-MS (matrix: glycerol) 477 (M+Na)+, 455(M+H)+ ; ^{1}H-NMR (D_2O), δ(ppm): 1.96 (2H, dt, J=8.4, 7.0 Hz,βCH_2- glu), 2.25 (2H,t, J=8.4 Hz,γCH_2-glu), 3.22 and 3.36 (1H,dd, J= 15.4,9.1 Hz and 1H, dd, J= 15.4,4.2 Hz,-CH_2-cys), 3.53 (2H,s,-CH_2 gly), 3.67 (1H, t,J=7.0 Hz, >CH- glu), 4.30(1H,dd, J= 9.1, 4.2 Hz, >CH- cys), 6.50 (1H,d, J=2.9Hz, H-3), 7.06 (1H,s, H-7), 7.25 (1H,d, J=2.9 Hz, H-2); ^{13}C-NMR (D_2O), δ(ppm): 26.75 (t,βCH_2- cys), 32.06 (t,β CH_2- glu), 35.11 (t,γCH_2- glu), 44.02 (t,-CH_2- gly),54.78 (d,>CH- glu), 54.99 (d,>CH- cys) 100.65 (d,C-7), 100.86 (d, C-3), 107.44 (s, C-4), 125.09 (s, C-9), 125.75 (d, C-2), 130.26 (s, C-5), 141.84 (s, C-6), 142.45 (s, C-8), 172.44 (s, -CO-), 174.49 (s, -CO-), 175.43 (s, -CO-), 176.93 (s, -CO-).

Conjugation of 5,6-dihydroxy-1-methylindole (3) with 4a and 4b:

1)by tyrosinase

To a solution of 3 (150 mg, 0.92 mmol) and the thiol (3.68 mmol) in 150 ml of 0.025 M phosphate buffer pH 6.8 a solution of mushroom tyrosinase (86,000 units) in 10 ml of the same buffer, was added under stirring. After 2 h, the reaction was stopped by addition of $Na_2S_2O_4$ and worked up as follows, depending on the thiol used.

In the case of L-cysteine ethyl ester (4a) , the reaction mixture was repeatedly extracted with ethyl acetate and the combined organic layers were washed with water, dried over Na_2SO_4 and evaporated to dryness. The residue so obtained was acetylated with acetic anhydride (2 ml) and pyridine (100 μl) at room temperature, for 12 h. Removal of the solvent left an oil which was purified by TLC on silica with ethyl acetate/benzene (6:4) to give, besides 5,6-diacetoxy-1-methylindole (11 mg, 5% yield), 4-(2-acetylamino-2-carboethoxy-ethylthio) -5,6-diacetoxy-1-methylindole (5a) (250 mg, 62% yield), colourless needles from ethyl acetate, m.p.107-108 °C; $[\alpha]_D^{25°}$=-20.4°(c=0.75, $CHCl_3$); λmax(EtOH) 304 nm (logε 4.0); EIMS m/e: 436(M+,10), 394 (12), 352 (30), 279 (8), 237 (20), 195 (100), 162 (28), 158 (68), 137 (12), 115 (25), 111 (14); (found M+ 436.1300, $C_{20}H_{24}N_2O_7S$ requires 436.1304); ^{1}H-NMR ($CDCl_3$) δ(ppm): 1.23 (3H, t, J=7.3 Hz, -CH_3), 1.57 (3H, s, -NHCOCH_3), 2.31 (3H, s,-OCOCH_3), 2.42 (3H, s, -OCOCH_3), 3.34 and 3.58 (1H,dd, J=15.1, 4.6 Hz, and 1H, dd, J=15.1, 4.1 Hz, -SCH_2-), 3.72 (3H,s,-NCH_3), 4.15(2H,q, J=7.3 Hz, -OCH_2-), 4.77 (1H, ddd, J=8.2, 4.6, 4.1 Hz, -CH<), 6.48 (1H, d, J= 8.2 Hz, -NHCO-), 6.62 (1H,dd, J=3.7, 0.8 Hz, H-3), 7.10 (1H, d, J=3.7 Hz, H-2), 7.15 (1H, d, J=0.8 Hz, H-7); ^{13}C-NMR ($CDCl_3$), δ(ppm): 14.02 (q, -CH_3), 20.40 (q, -OCOCH_3), 20.61 (q, -OCOCH_3), 22.21 (q, -NHCOCH_3), 33.05 (q, -NCH_3), 35.92 (t, -SCH_2-), 53.40 (t, -OCH_2-), 61.58 (d, -CH<), 100.81 (d, C-7), 104.53 (d,C-3), 119.06 (s,C-4), 128.60 (s, C-9), 131.14 (d, C-2), 133.21 (s, C-5), 137.43 (s, C-6), 138.06 (s, C-8), 168.83 (s, -OCOCH_3), 169.31 (s, -OCOCH_3), 169.89 (s, -NHCOCH_3), 169.96 (s, -COOCH_2CH_3).

In the case of N-acetyl-L-cysteine (4b), the reaction mixture was acidified to pH 2.5 with H_3PO_4 and repeatedly extracted with ethyl acetate. The combined organic layers were washed with water, dried over Na_2SO_4 and evaporated to dryness in vacuo. The residue,dissolved in MeOH, was treated with an excess of ethereal diazomethane for 12 h at room temperature. After removal of the solvent, the residue was fractionated by TLC with $CHCl_3$/MeOH (97:3) and the band at Rf 0.50 was further purified by preparative HPLC to give 4-(2-acetylamino-2-carbomethoxy-ethylthio)-5,6-dimethoxy-1-methylindole (5b), (105 mg, 32% yield),as an oil, UV λmax (EtOH) 306, 294 (shoulder) nm; $[\alpha]_D^{25°}$ =+13.8° (c=1.62,$CHCl_3$); EIMS m/e: 366 (M+, 56), 307 (19), 292 (12), 248 (40), 236 (25), 223 (100), 208, (80), 176 (16), 164 (27), 144 (52); (found M+ 366.1241, $C_{17}H_{22}N_2O_5S$ requires 366.1249); H-NMR ($CDCl_3$), δ(ppm):1.74 (3H,s, -NHCOCH$_3$), 3.19 and 3.49 (1H, dd,J=14.1, 4.6 Hz and 1H, dd, J= 14.1, 4.2 Hz,-SCH$_2$-),3.54 (3H,s, -OCH$_3$),3.72 (3H,s,-NCH$_3$), 3.89 (3H,s, -OCH$_3$), 3.91(3H,s, -OCH$_3$),4.78 (1H, ddd, J=7.7,4.6,4.2 Hz,-CH<), 6.52 (1H, dd, J=3.3, 0.8 Hz, H-3), 6.78 (1H, d,J=0.8 Hz, H-7), 7.00 (1H, d, J= 3.3 Hz, H-2), 7.08 (1H,d, J= 7.7 Hz,-NHCO-); ^{13}C-NMR ($CDCl_3$) δ(ppm): 22.56 (q,NHCOCH$_3$),33.09 (q,-NCH$_3$), 36.90 (t,-SCH$_2$-), 52.17 (q, -OCH$_3$), 52.41 (q,-OCH$_3$), 56.31 (q,-OCH$_3$), 61.85 (d, -CH<), 94.25 (d, C-7), 100.57 (d, C-3), 118.21 (s, C-4),125.11 (s, C-9), 128.43 (d, C-2), 132.54 (s, C-5), 145.51 (s, C-6), 149.96 (s, C-8), 169.85 (s,-NHCOCH$_3$), 170.84 (s, -COOCH$_3$).

2) by Fe^{2+}-EDTA /H_2O_2

A solution of 3 (100 mg,0.6 mmol), $FeSO_4 \cdot 7H_2O$ (17 mg,0.06 mmol),EDTA·2Na (46 mg,0.12 mmol),in 65 ml of water was adjusted to pH 7.0 with crystals of $Na_2HPO_4 \cdot 12H_2O$.To the vigorously stirred solution were added the thiol 4a/b (0.24 mmol) and 1 min later 12 μl of 9.7 M H_2O_2 (0.12 mmol). The additions of the same amounts of the thiol and H_2O_2 were repeated 5 times at 10 min intervals. Ten min after the final addition of H_2O_2 the resulting brown reaction mixture was worked up as in the tyrosinase-catalyzed reaction, depending on the thiol used.

In the case of 4a, 48 mg of 5a (18% yield) and 59 mg of the starting material as 5,6-diacetoxy-1-methylindole (39% yield) were obtained.

In the case of 4b, 35 mg of 5b (16% yield) and 7 mg of the starting material as 5,6-dimethoxy-1-methylindole (6% yield) were obtained.

3) by persulphate

To a stirred solution of 3 (100 mg, 0.6 mmol) and the thiol (0.6 mmol) in 100 ml of 0.1 M phosphate buffer, pH 7.5 , a solution of $(NH_4)_2S_2O_8$ (140 mg, 0.6 mmol) in 25 ml of the same buffer was added dropwise over 15 min. The mixture was allowed to stand for additional 90 min and then was worked up as in the tyrosinase-catalyzed reaction depending on the thiol used.

In the case of 4a, 45 mg of 5a (17% yield) and 63 mg of 5,6-diacetoxy-1-methylindole (42% yield) were obtained.

In the case of 4b, 45 mg of 5b (20% yield) and 26 mg of 5,6-dimethoxy-1-methylindole (22% yield) were obtained.

4) by $CuSO_4$

To a stirred solution of 3 (100 mg, 0.6 mmol) and 4b (400 mg, 2.4 mmol) in 200 ml of 0.5 M Tris buffer[21], pH 7.5, a solution of $CuSO_4 \cdot 5H_2O$ (300 mg, 1.2 mmol) in 20 ml of the same buffer was added dropwise over a period of 10 min. The reaction mixture was allowed to stand for additional 5 min and then treated with $Na_2S_2O_4$, acidified to pH 2.5 with HCl and extracted repeatedly with ethyl acetate. The combined organic layers were washed with 0.1 M HCl and taken to dryness. Purification of the residue as above afforded 11 mg of 5b (5% yield) and 42 mg the starting material as 5,6-dimethoxy-1-methylindole (36% yield).

When thiol 4a was used, no formation of the corresponding adduct 5a was observed.

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REFERENCES AND NOTES

1. G.Prota, J.Invest. Dermatol., 75, 122, 1980.
2. G.Prota in "Natural sulfur compounds", eds. D.Cavallini, G.E.Gaull, V.Zappia, Plenum Publ. Corporation, New York, 1980, p. 391
3. S.Rothman,H.Krysa, A.M.Smiljanic, Proc. Soc. Exper. Biol. Med., 62, 208, 1946.
4. P.Flesch, Proc. Soc. Exper. Biol. Med., 70, 136, 1949.

5. A.B.Lerner, T.B.Fitzpatrick, E.Calkins, W.H.Summerson, J.Biol.Chem.,187, 793, 1950.
6. S.Ito, A.Palumbo, G.Prota, Experientia, 41, 960, 1985.
7. S.Ito, G.Prota, Experientia, 33, 1118, 1977.
8. R. Carstam, C.Edner, C.Hansson, C.Lindbladh, H.Rorsman, E.Rosengren, Acta Derm. Venereol., (Stockh), 66, 1, 1986.
9. S.Bouchilloux, A.Kodja, Bull.Soc.Chim.Biol. (Paris), 42, 1045, 1960.
10. H.S.Mason, E.W.Peterson, Biochim.Biophys.Acta, 111, 134, 1965.
11. G.Prota, G.Scherillo, E.Napolano, R.A.Nicolaus, Gazzetta,97, 1451, 1967.
12. N.N.Nkpa, M.R.Chedekel, J.Org.Chem., 46, 213, 1981.
13. S.Ito, Bull. Chem. Soc. Japan, 56, 365, 1983.
14. J.M.Bowness, R.A.Morton, Biochem.J., 53, 620, 1953.
15. K.Lerch in "Metal ions in biological systems", ed. H.Sigel, Marcel Dekker Inc., New York, Basel, 1981, p.143.
16. F.Minisci, A. Citterio, C. Giordano, Acc.Chem.Res.,16,27, 1983.
17. C. Walling, Acc. Chem. Res., 8, 125, 1975.
18. This quinone has been postulated by several authors as an intermediate in the oxidative conversion of 1 to melanin but, up to the present, its actual existence has never been substantiated by unambiguous experimental evidence.
19. J.D.Benigni, R.L.Minnis, J. Heterocyclic.Chem.,2, 387, 1965.
20. M.G.Corradini, A.Napolitano, G.Prota, Tetrahedron, 42, 2083, 1986.
21. 2-Amino-2-hydroxymethyl-1,3-propandiol.