thus compete with ethanol for binding to the same catalytic site.

The increased turnover number in the presence of deoxycholate has been attributed to a modification by this steroid of a rate-limiting conformational change involved in the reaction mechanism of the dehydrogenase[5]. Such an increase is similar to that observed with the enzyme modified by reductive alkylation: since the 2 effects are not additive, they are probably dependent on the same mechanism, in which 1 or more amino groups, protected from methylation by the binding with the coenzyme, could be involved. Therefore the interaction of rat liver alcohol dehydrogenase with deoxycholate and the methylation procedure could promote the same general conformational modification, which seems to be the basic rationale of the observed kinetic changes.

1 Acknowledgment. We thank Professor A. Guerritore for his help and advice. This work was supported by a grant from the Italian Consiglio Nazionale delle Ricerche.
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Co-polymerization of dopa and cysteinyldopa in melanogenesis in vitro[1]

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Summary. Tyrosinase oxidation of dopa in the presence of varying amounts of 5-S-cysteinyldopa led to the production of pigments whose chemical and physical (colour and solubility) properties are intermediate between those of pure eumelanin- and phaeomelanin-type pigments.

Recent advances[3,4] in the chemistry of melanogenesis have revealed that melanin pigmentation of hair, skin and eye results mainly from 2 separated but biogenetically interrelated classes of pigments: the black-brown, insoluble, nitrogenous eumelanins which derive from the enzymic (tyrosinase) oxidation of tyrosine via dopa, and the sulphur-containing alkali-soluble phaeomelanins, ranging from yellow to reddish-brown, which arise by a diversion from the eumelanin pathway through the intervention of cysteine or related sulphydryl compounds[5]. Chemical and biochemical studies provide evidence that a major intermediate in the biosynthesis of phaeomelanins is 5-S-cysteinyldopa (sulphur content: 10.1%) which is formed by the addition of cysteine to enzymically produced dopaquinone. The high content (9–10%) of sulphur in the phaeomelanins isolated from reddish hair from various mammals, including man[6], is quite consistent with the proposed pathway. In many cases, however, no strict borderline exists between eumelanins and phaeomelanins with respect to chemical and physical properties. It is, for example, typical of all eumelanins so far isolated that they contain sulphur in various percentages from 1% to as much as 5%, suggesting a possible intermeshing between eumelanin and phaeomelanin pathways[7].

To test this hypothesis, we have carried out a series of model reactions simulating the intermeshing of the 2 pigmentary pathways in melanogenesis by the enzymic oxidation of dopa in the presence of varying amounts of 5-S-cysteinyldopa (abbreviated as cys-dopa).

Materials and methods. Co-oxidation of dopa and cys-dopa. A mixture of L-dopa and cysdopa[8], the total amount being 0.5 mmoles in various ratios (see table) in 0.05 M sodium phosphate buffer, pH 6.8 (40 ml) was vigorously stirred at 37 °C, under an oxygen current, in the presence of mushroom tyrosinase (4 mg, 2230 units/mg, from Sigma Chemical Co.). After 4 hr the oxidation was stopped by acidification to pH 3.5 with 2M HCl and the mixture was kept at room temperature for 1 h. The resulting precipitate was then collected by centrifugation, washed with 1% acetic acid (3 times), acetone (twice) and dried in vacuo over P_2O_5 overnight.

Degradation with 57% HI[6]. A mixture of the biosynthetic melanin (25 mg) and red phosphorus (5 mg) in 3 ml of freshly distilled 57% HI was refluxed for 24 h. The reaction mixture was then evaporated to dryness under reduced pressure and the residue, taken up in water, was fractionated by chromatography on Whatman 3MM paper (eluents: 1-butanol-acetic acid-water, 60:15:25 and 0.08M HCl) to give 3-hydroxy-4-aminophenylalanine, β-6-(4-hydroxybenzothiazolyl)alanine and 2-methyl-β-6-(4-hydroxybenzothiazolyl)alanine identified by comparison of their chromatographic and spectral (UV) properties with those of authentic samples.

Alkaline fusion[6]. A mixture of the biosynthetic melanin (20 mg), NaOH (100 mg), $Na_2S_2O_4$ (15 mg) and water (0.3 ml) was heated in a platinum crucible at 280–300 °C for 10 min. After cooling, the fused mass was worked up according to the procedure already described[9], and ana-

Physical and chemical properties of melanins obtained by enzymic oxidation of dopa and/or cys-dopa

Substrate(s)[a] Dopa	Cys-dopa	Yield	Colour	Solubility in 0.1 M NaOH	Composition N (%)	S (%)	N:S ratio Found	(Calculated[b])	Degradation products 57% HI[c]	Alkaline fusion[d]
100%	0%	50 mg	Black	None	8.97	-	-	(2:0)	−	++++
95%	5%	50 mg	Black	None	9.22	1.95	10.8:1	(21:1)	+	++++
80%	20%	66 mg	Dark brown	Low	9.49	3.30	6.58:1	(6:1)	++	++++
60%	40%	64 mg	Light brown	High	9.61	5.98	3.68:1	(3.5:1)	++	++
40%	60%	60 mg	Yellowish brown	High	10.03	9.04	2.54:1	(2.7:1)	+++	+
0%	100%	65 mg	Reddish brown	Very high	10.23	10.51	2.23:1	(2:1)	++++	−

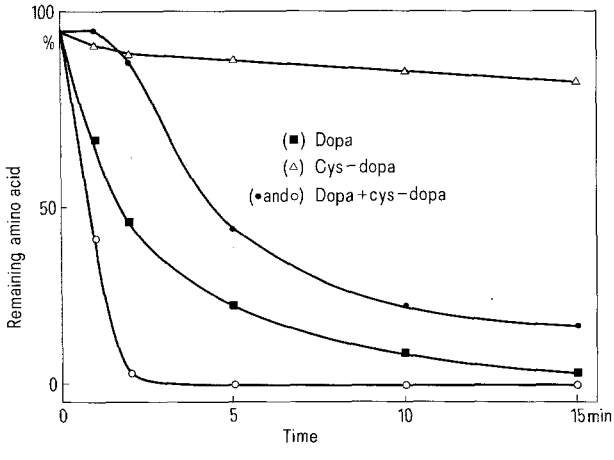
[a] All reactions were carried out on a total amount of 0.5 mmoles of substrate(s) as described in the text. [b] Calculated on the assumption that the products retained the original molar ratios of dopa to cys-dopa. [c] Products analyzed were 3-hydroxy-4-aminophenylalanine, β-6-(4-hydroxybenzothiazolyl)alanine and 2-methyl-β-6-(4-hydroxybenzothiazolyl)alanine. [d] Product analyzed was 5,6-dihydroxyindole.

lyzed for the presence of 5,6-dihydroxyindole by paper chromatography in 1-butanol-acetic acid-water (60:15:25) using as spray reagents 3% ethanolic $FeCl_3$ and aqueous Fast red B salt (Fluka).

Results and discussion. In preliminary experiments the course of tyrosinase oxidation of an equimolar mixture of dopa and cys-dopa was studied. The enzymic oxidations of the mixtures, dopa alone and cys-dopa alone were compared by analyzing the amounts of dopa and/or cys-dopa remaining in the reaction mixtures. As shown in the figure, when compared with dopa, cys-dopa itself is a very poor substrate for tyrosinase. However, in the presence of an equimolar quantity of dopa, the rate of decrease of cys-dopa was dramatically accelerated (> 0.18 μmoles/min/ml), even exceeding that of dopa alone (initial rate: 0.11 μmoles/min/ml), while dopa in the mixture started to disappear after a lag time of 1–2 min. These results indicate that tyrosinase first oxidizes dopa to dopaquinone which in turn oxidizes cys-dopa to cys-dopaquinone and itself is reduced back to dopa. The rapid decrease of cys-dopa may be attributed also to the pathway proposed by Prota et al.[10] that cys-dopaquinone cyclizes to give the corresponding o-quinonimine derivative which then catalyzes the conversion of cys-dopa.

We next prepared pigments by enzymic oxidation of mixtures of dopa and cys-dopa in various ratios. The table summarizes the physical properties (colour and solubility), elemental analyses and results of chemical degradations of these pigments. 5,6-Dihydroxyindole is a typical degradation product obtained by alkaline fusion of natural eumelanin-type pigments[9]. Reductive degradation with 57% HI of natural phaeomelanin-type pigments is known to give 3-hydroxy-4-aminophenylalanine and 4-hydroxybenzothiazolylalanines[6]. The pigments formed by the enzymic oxidation of dopa in the presence of varying amounts of cys-dopa had chemical and physical properties intermediate between those of pure eumelanin- and phaeomelanin-type polymers obtained from 100% of dopa and cys-dopa, respectively. Furthermore, the nitrogen to sulfur ratios of the pigments are in fairly good agreements with the calculated values.

The present results clearly indicate that dopa and cys-dopa can co-polymerize in any ratio by tyrosinase oxidation in vitro. Previously, we showed that the enzymic oxidation of dopa in the presence of an excess of cysteine gave cysteinyldopas in a high yield, while no pigments were formed[8], indicating that the addition of cysteine to dopaquinone can proceed much faster that the intramolecular cyclization of dopaquinone to cyclodopa (leucodopachrome). In addition, it is known that active melanocytes produce cysteinyldopas as well as dopa[11]. These facts, together with the present findings suggest that eumelanin-type pigments produced in melanogenesis in vivo may be regarded as co-polymers derived from dopa and cys-dopa in various ratios, as long as they contain sulfur.



Reaction rates of tyrosinase oxidation of dopa, dopa plus cys-dopa and cys-dopa. The reaction mixture contained: either 0.9 μmoles of L-dopa, 0.9 μmoles of L-dopa plus 0.9 μmoles of cys-dopa or 0.9 μmoles of cys-dopa, and 48 μg of mushroom tyrosinase in 3 ml of 0.05 M sodium phosphate buffer, pH 6.8. At 1, 2, 5, 10 and 15 min after the reaction had been started, aliquots of the reaction mixture were removed and mixed with a pH 2.2 buffer containing L-alanine as an internal standard and $Na_2S_2O_5$ as a reducing reagent. The amounts of dopa and/or cys-dopa in the mixture were determined with a JEOL JLC-6AH amino acid analyzer using a pH 4.25 buffer as the eluent.

1 Acknowledgment. This work was supported in part by a grant from CNR, Rome, within the frame of the project 'Controllo della crescita neoplastica'.
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