A new dopachrome-rearranging enzyme from the ejected ink of the cuttlefish *Sepia officinalis*

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A melanogenic enzyme catalysing the rearrangement of dopachrome has been identified in the ejected ink of the cuttlefish *Sepia officinalis*. This enzyme occurs as a heat-labile protein which co-migrates with tyrosinase under a variety of chromatographic and electrophoretic conditions. On SDS/PAGE it shows like a single band with an approx. molecular mass of 85 kDa. The enzyme possesses high substrate specificity, acting on L-dopachrome (K_m = 1 mM at pH 6.8) and on L-α-methyldopachrome, but not on D-dopachrome, L-dopachrome methyl ester, dopaminochrome and adrenochrome. Significant inhibition of the catalytic activity was observed with tropolone and L-mimosine. H.p.l.c. analysis of the enzyme-catalysed rearrangement of L-dopachrome revealed the quantitative formation of the decarboxylated product, 5,6-dihydroxyindole. These results point to marked differences between melanogenesis in cephalopod pigment cells and in melanocytes, which may have important implications in relation to the use of sepiomelanin as a model for studies of mammalian melanins.

INTRODUCTION

Over the last few years the advent of molecular biology has valuably contributed to the dissecting of the developmental processes underlying melanin pigmentation (Prota, 1992). Families of genes with significant sequence similarity have been isolated and shown to encode melanogenic proteins with similar structural characteristics but with different functions (Hearing and Tsukamoto, 1991; Halaban and Moellman, 1992; Takeuchi, 1992; Kwon, 1993; Shibahara, 1993). To a considerable degree, interpretation of these studies has been driven by previous chemical and biochemical investigations, that have uncovered an array of enzymes and cofactors intervening at various sites of the pigment pathway (Prota, 1993). As a result, melanogenesis appears now as a multifaceted process whose outcome depends upon a concert of regulatory factors acting on a genetic basis and/or in response to contingent conditions.

The primary requirement for the whole process to occur is the presence of the copper enzyme tyrosinase, which is now known to be encoded at the *albino* (*c*) locus on chromosome 7 in the mouse (Kwon et al., 1989; Yamamoto et al., 1989; Takeuchi et al., 1993). This enzyme catalyses the initial rate-determining step of melanogenesis, i.e. the hydroxylation of tyrosine to dopa (3,4-dihydroxyphenylalanine) and its oxidation to dopaquinone.

Of the enzymes acting on the subsequent steps, dopachrome tautomerase (DT) has attracted the keenest interest. First detected in Cloudman-melanoma cells by Korner and Pawelek (1980), and termed 'dopachrome conversion factor', this enzyme was later described by various laboratories in both melanotic and amelanotic melanoma-cell lines, as well as in extracts of melanocyte-containing tissues from mice, hamsters and humans (Barber et al., 1984; Lamoreoux et al., 1986; Aroca et al., 1990; Pawelek, 1991; Townsend et al., 1992).

DT has been shown to correspond to TRP-2, a member of a family of tyrosinase-related proteins which is encoded at the *slaty* locus in mice (Jackson et al., 1992a; Tsukamoto et al., 1992a). A study of the expression of TRP-2 by *in situ* hybridization during embryonic development has revealed the presence of this protein in the developing forebrain (Jackson et al., 1992b). More recently, DT activity has been reported also for the mouse *brown* (*b*) locus protein (Winder et al., 1993).

DT specifically acts on a crucial branching point of melanogenesis distal to tyrosinase, namely the rearrangement of dopachrome (Scheme 1). This is a rather slow reaction that, at physiological pH values, proceeds with concomitant decarboxylation, to yield mainly 5,6-dihydroxyindole (DHI), along with small amounts of 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Upon the action of the enzyme, the chemical course of the reaction is diverted, whereby the non-decarboxylative tautomerization to DHICA becomes the dominant route.

An enzyme acting on the rearrangement of dopachrome has also been reported to occur in the haemolymph of the tobacco hornworm (*Manduca sexta*) (Aso et al., 1989; Sugumaran and Semensi, 1991). This enzyme, originally named 'dopaquinone-imine conversion factor', differs from mammalian DT in that it catalyses the decarboxylative rearrangement of dopachrome, leading to DHI rather than to DHICA.

We now report that the ejected ink of *Sepia officinalis* contains another melanogenic enzyme which catalyses the conversion of dopachrome into DHI.

EXPERIMENTAL

Materials

L-Dopa, D-dopa, dopamine, L-dopa methyl ester, adrenaline, tropolone and L-mimosine were obtained from Sigma Chimica (Milan, Italy). Silver(I) oxide, L-α-methyldopa and 5-hydroxyindole were from Fluka (Buchs, Switzerland). 5-Hydroxyindole-2-carboxylic acid was purchased from Aldrich. All other chemicals were of the highest purity available. DHI, DHICA and 2-methyl-

Abbreviations used: DT, dopachrome tautomerase; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; 2-MeDHI, 2-methyl-5,6-dihydroxyindole; DTT, dithiothreitol.

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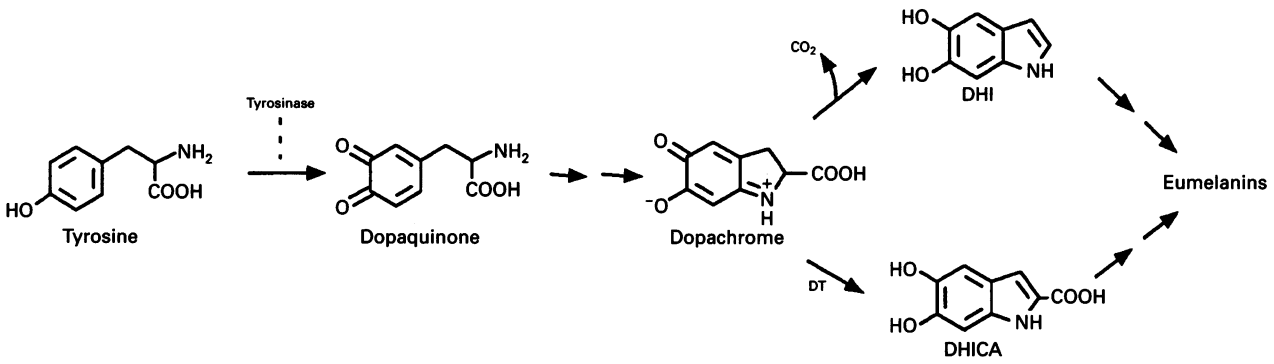


Scheme 1 Schematic outline of melanogenesis highlighting the effect of DT on the rearrangement of dopachrome

5,6-dihydroxyindole (2-MeDHI) were prepared as described by Benigni and Minnis (1962).

Animals

Specimens of *Sepia officinalis* were collected in the bay of Naples. The animals were killed by decapitation, and the whole ink present in the ink sac was used.

Enzyme preparation and assay

The crude ink of *Sepia* was ultracentrifuged at 50000 rev./min for 1 h to remove melanin. The supernatant, dialysed against deionized water, was freeze-dried. The residue (225 mg) was chromatographed on a column (1.9 cm × 35 cm) of DEAE-Bio-Gel agarose (100–200 mesh) equilibrated in 10 mM sodium phosphate, pH 6.8. The enzyme was eluted with a linear gradient of 0–0.4 M NaCl in the starting buffer. Fractions of about 4.5 ml were collected and analysed for tyrosinase as well as for catalytic effects on dopachrome rearrangement. Fractions 63–73, exhibiting the highest specific enzymic activity towards L-dopachrome, were pooled, dialysed exhaustively against water and freeze-dried. The enzyme preparation thus obtained (30 units/mg of protein) was used throughout this study. One unit of dopachrome rearranging activity was defined as the amount of enzyme that catalyses the conversion of 1 μmol of dopachrome/min under the above conditions (Palumbo et al., 1991). The protein content was determined by the method of Lowry et al. (1951), with crystalline BSA as standard.

Tyrosinase activity was determined spectrophotometrically at 27 °C by measuring the rate of formation of dopachrome from L-tyrosine (0.4 mM) in 0.05 M phosphate buffer, pH 6.8. One unit of tyrosinase was defined as the amount of enzyme which is required to produce 1 μmol of dopachrome/min under the above conditions (Palumbo et al., 1985, 1990).

PAGE

Gel electrophoresis under native and denaturing conditions was performed using a Pharmacia PhastSystem apparatus. Native PAGE was carried out on homogeneous 7.5 gels using native buffer strips and run at 80 V·h. The protein bands were stained with the fast Coomassie Blue method as described by the manufacturer. For tyrosinase activity staining, the gel was washed with 0.5 M phosphate buffer, pH 6.6 at 4 °C for 1 h and then treated at 37 °C with 0.15% dopa in 0.1 M phosphate buffer, pH 6.8, until appearance of dark brown colouration due to melanin formation.

For electrophoresis under denaturing conditions, the samples in 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA were heated at 100 °C for 5 min in the presence of 1% dithiothreitol (DTT) and 2.5% (w/v) SDS. Electrophoresis was carried out on homogeneous 7.5% gels using 0.55% SDS buffer strips and run at 60 V × h.

Proteins were detected by fast Coomassie Blue staining. The low-molecular-mass calibration kit from Pharmacia was used as the standard molecular-mass marker proteins.

Preparation of dopachrome and related compounds

L-Dopachrome was prepared by oxidation of L-dopa with silver(I) oxide, as previously described (Palumbo et al., 1987). L-Dopa (0.5 mM) in 0.05 M sodium phosphate buffer, pH 6.8 (4 ml), was vigorously shaken with silver(I) oxide (4 mg) for 4 min, and the final suspension was filtered through a 0.45-μm-pore-size Millipore filter. The resultant solution was treated batchwise with Chelex 100 (100–200 mesh; Na^+ form; Bio-Rad) to remove traces of silver. This procedure resulted in a more than 85% conversion of dopa into dopachrome as determined spectrophotometrically. Aliquots (1.0 ml) of the dopachrome solution were immediately used for kinetic experiments. D-Dopachrome, L-α-methyldopachrome, dopaminochrome, L-dopachrome methyl ester were prepared similarly from D-dopa, L-α-methyldopa, dopamine and L-dopa methyl ester, respectively. Adrenochrome was prepared as described by Sobotka and Austin (1951).

Kinetic experiments

The catalytic effect of *Sepia* ink on dopachrome rearrangement was determined spectrophotometrically by monitoring the absorbance decrease at 475 nm (ϵ 3600 $M^{-1} \cdot cm^{-1}$; Mason, 1948). A Beckman DU-65 spectrophotometer interfaced to a IBM PC XT model 286 computer was used. Dopachrome (0.4 mM in 0.05 M phosphate buffer, pH 6.8) was allowed to rearrange in a rubber-capped cuvette under N_2 (to prevent oxidation of the resultant indoles). The temperature was maintained at 27±0.1 °C with circulating water in the cell holder. Aliquots of the partially purified enzyme fraction were added to the reaction mixture with a syringe, care being taken to ensure rapid and complete mixing of the reagents. When necessary, aliquots of stock solutions of additives (inhibitors, metal chelators, etc.) were added to the reaction mixture with a syringe prior to the enzyme.

Inhibition experiments with phenylthiourea or dipyridyl were carried out by pre-incubating the enzyme with the inhibitor or

appropriate solvent blanks for 30 min at 27 °C. Aliquots of the incubation mixture were then withdrawn and added to the dopachrome solution for kinetic measurements.

Initial rates were determined in triplicate in the linear portion of the curve during the first 20 s of the reaction. Experiments at various pH values were performed in phosphate buffers with the conductivity adjusted to 13 mS by addition of KCl. The enzymic rearrangement of dopachrome-related aminochromes was carried out as above.

Product analysis

H.p.l.c. analysis of the dopachrome and methyldopachrome rearrangement products was carried out on a Gilson model 302 instrument, using a 25 cm S5 ODS2 Spherisorb column. The mobile phase was 0.05 M citric acid/acetonitrile (9:1, v/v) adjusted to pH 4 with NaOH, and the flow rate was maintained at 1 ml/min. When the rearrangement reaction was completed, as judged by the fading of the red chromophore, an aliquot of the solution was directly injected for analysis of DHI, DHICA and 2-MeDHI. Detection of the compounds was carried out at 280 nm with a Gilson model 116 u.v. detector. Measurement of retention times and peak areas and comparison with external calibration curves for DHI, DHICA, 2-MeDHI allowed quantitative analysis of the reaction mixtures.

RESULTS

Figure 1 shows the effect of the crude, melanin-free ink of *Sepia officinalis* on the rearrangement of L-dopachrome in phosphate buffer at pH 6.8, as monitored spectrophotometrically at 475 nm. In the presence of added ink, a marked acceleration of the kinetics of the reaction was observed, accounting for about 0.4 unit of dopachrome-rearranging activity/ml of ink. Heating the ink sample at 85 °C for 20 min completely suppressed the catalytic effect, whereas chromatography on Chelex-100, to remove metal impurities, had no significant effect.

On PAGE analysis the melanin-free *Sepia* ink showed as an ill-defined pattern of proteins comprising a major tyrosinase fraction (Prota et al., 1981), as revealed by dopa staining. Under denaturating conditions with SDS, however, resolution to a major band was observed, with an apparent molecular mass of 85 kDa (Figure 2).

Attempts to separate the dopachrome-rearranging enzyme from tyrosinase were unsuccessful under a variety of conditions, including chromatography on Sephacryl S-300 and h.p.l.c. on a polyethyleneimine column (Townsend et al., 1992). Chromatography of the melanin-free ink on a DEAE-Bio-Gel agarose column afforded a major peak possessing dopachrome-rearranging activity which was only poorly resolved from the tyrosinase peak (Figure 3).

The enzyme preparation obtained by DEAE-Bio-Gel agarose

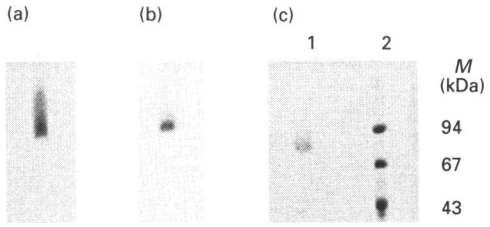


Figure 2 PAGE of crude melanin-free ink of *Sepia officinalis*

(**a**) Native PAGE (gel stained with Coomassie Blue); (**b**) native PAGE (gel stained with dopa); (**c**) SDS/PAGE (gel stained with Coomassie Blue). Lane 1, crude melanin-free ink; lane 2, molecular-mass (*M*) marker proteins: phosphorylase *b* (94 kDa), BSA (67 kDa) and ovalbumin (43 kDa). Details are given in the Experimental section.

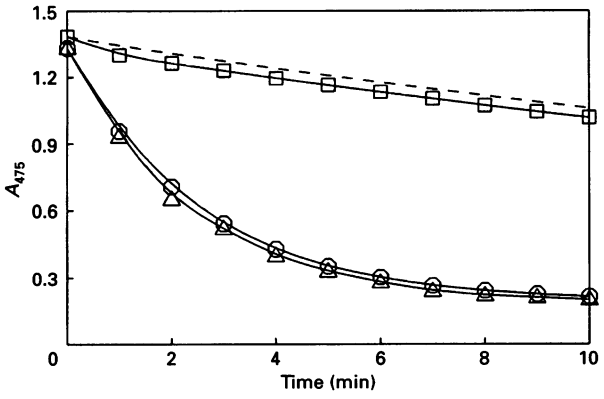


Figure 1 Effect of melanin-free ink of *Sepia officinalis* on the rearrangement of L-dopachrome

L-Dopachrome (0.4 mM) was allowed to rearrange in 0.05 M phosphate buffer, pH 6.8, at 27 °C. ----, control; ○, +2 mg/ml freeze-dried melanin-free ink; □, +2 mg/ml heated melanin-free ink; △, +2 mg/ml melanin-free ink after passage on Chelex-100 resin.

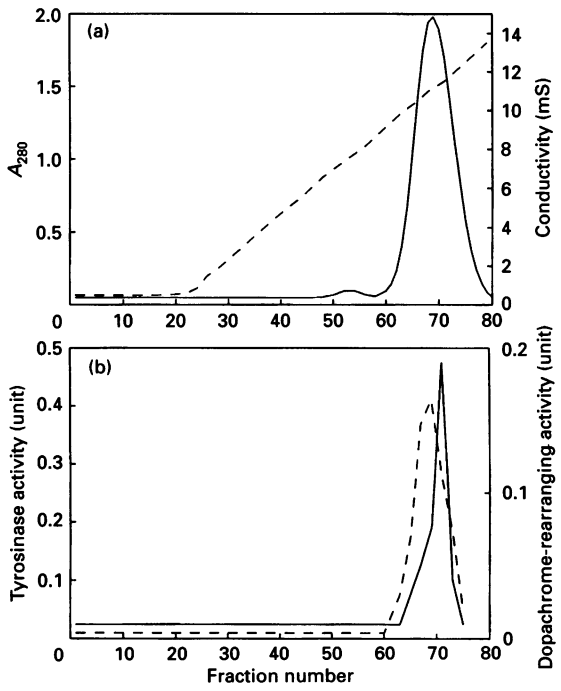
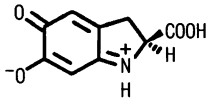
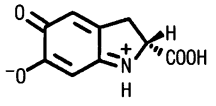
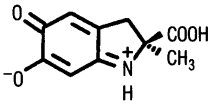
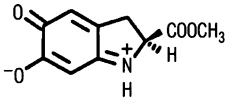
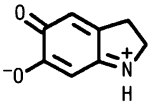
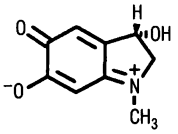


Figure 3 DEAE-Bio-Gel agarose chromatography of the melanin free-ink of *Sepia officinalis*

The freeze-dried melanin-free ink (225 mg) was loaded on a column (1.9 cm × 35 cm) of DEAE-Bio-Gel agarose (100–200 mesh) equilibrated in 10 mM sodium phosphate, pH 6.8. (**a**) The enzyme was eluted with a linear gradient (----) of 0–0.4 M NaCl. Protein elution was followed spectrophotometrically at 280 nm (——). (**b**) Tyrosinase (----) and dopachrome-rearranging (——) activities were determined in the eluted fractions as detailed in the Experimental section.

Table 1 Catalytic effects of *Sepia* enzyme on the rate of rearrangement of L-dopachrome and related aminochromes

The aminochromes (0.4 mM) were allowed to rearrange at 27 °C in 0.05 M phosphate buffer, pH 6.8, under N_2 in the presence and in the absence of *Sepia* enzyme (0.1 unit/ml). All experiments were carried out at least in triplicate. Catalytic effects are expressed as ratios of the initial rates in the presence (v) and in the absence (v_0) of added enzyme. Standard errors were within 4%.

Aminochrome	Structural formula	v/v_0
L-Dopachrome		9.5
D-Dopachrome		1.0
L-α-Methyldopachrome		7.6
L-Dopachrome methyl ester		1.0
Dopaminochrome		1.0
L-Adrenochrome		1.0

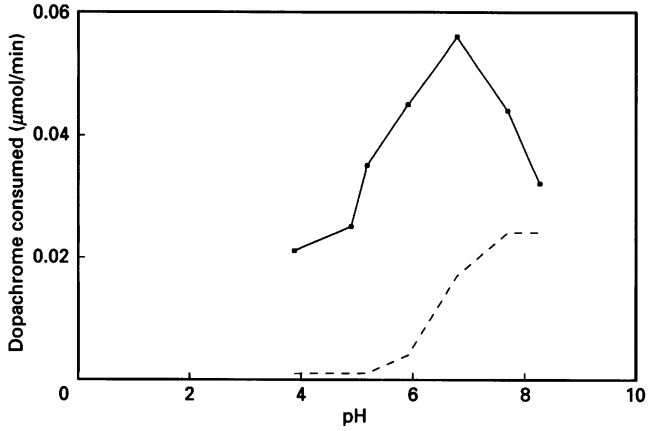


Figure 4 pH-dependence of the catalytic effect of partially purified enzyme on the rearrangement of L-dopachrome

L-Dopachrome (0.4 mM) was allowed to rearrange in 0.25 M phosphate buffer at 27 °C at various pH values in the presence (■) and in the absence (----) of 0.1 unit of enzyme/ml.

Table 2 Effect of metal chelators, tyrosinase and DT inhibitors on the catalytic effect of the dopachrome-rearranging enzyme from *Sepia*

Incubation conditions were as described in the Experimental section. Results are means ± S.D. for at least three experiments.

Inhibitor	Concn. (mM)	Dopachrome-rearranging activity (%)
None	–	100 ± 3
Tropolone	0.5	56 ± 4
L-Mimosine	0.5	60 ± 3
Phenylthiourea	0.5	99 ± 1
5-Hydroxyindole	1.0	98 ± 3
5-Hydroxyindole-2-carboxylic acid	1.0	99 ± 2
DHI	1.0	98 ± 3
DHICA	1.0	100 ± 1
2,2′-Dipyridyl	20.0	97 ± 4
EDTA	5.0	98 ± 2

chromatography exhibited a specific activity of 30 units/mg of protein as against a value of 20 units/mg for the crude melanin-free ink, and was preferably used throughout the present study.

A series of aminochromes structurally related to L-dopachrome were tested as substrates of the *Sepia* enzyme. Comparison of the rates of rearrangement of the aminochromes at 0.4 mM concentration in the presence and in the absence of added enzyme (0.1 unit/ml) showed that L-dopachrome is the best substrate, with a K_m of 1 mM, as determined in separate experiments. L-α-Methyldopachrome was also efficiently acted upon by the enzyme, whereas little or no catalytic effect was observed on D-dopachrome, L-dopachrome methyl ester, dopaminochrome and L-adrenochrome (Table 1).

Figure 4 shows the variations of the enzyme activity as a function of pH under conditions of constant ionic strength. The maximum catalytic activity was measured at a pH of about 6.8.

In another set of experiments the effect of a range of melanogenic inhibitors and metal chelators on the *Sepia* enzyme was investigated. At 0.5 mM concentration, tropolone and L-mimosine, which are effective inhibitors of tyrosinase (Khan and Andrawis, 1985), were found to produce significant inhibition of the enzyme-catalysed reaction. Phenylthiourea, another specific inhibitor of tyrosinase, had no detectable effect on the enzyme activity, even after prolonged pre-incubation. Hydroxylated and/or 2-carboxylated indoles such as 5-hydroxyindole, 5-hydroxyindole-2-carboxylic acid, DHI and DHICA, which have a marked inhibitory effect on mammalian DT (Aroca et al., 1991), as well as the iron chelator dipyridyl (Chakraborty et al., 1992) did not affect the kinetics of the enzymic rearrangement. No effect of other chelating agents, such as EDTA, was likewise observed (Table 2).

H.p.l.c. analysis of the products derived from enzyme-catalysed rearrangement of L-dopachrome revealed, after complete disappearance of the maximum at 475 nm, the almost exclusive formation of DHI (Figure 5). Control experiments showed that, under the reaction conditions used, DHICA is fairly stable and is by no means converted into DHI, assuring us that DHI formation was the result of the decarboxylative rearrangement

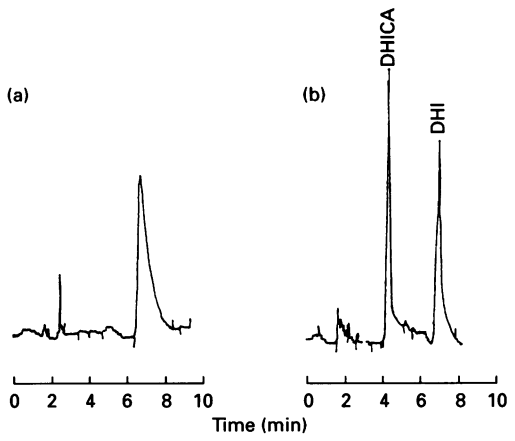


Figure 5 H.p.l.c. elution profile of the products formed by enzyme-catalysed rearrangement of L-dopachrome

L-Dopachrome (0.4 mM) was rearranged in the presence of *Sepia* enzyme in 0.05 M phosphate buffer, pH 6.8. Product analysis was carried out when all the dopachrome was decomposed. (**a**) *Sepia* enzyme (0.1 unit/ml); (**b**) Standard samples of DHI and DHICA.

of dopachrome. Analogously, the enzymic rearrangement of L-α-methyldopachrome afforded 2-methyl-5,6-dihydroxyindole (2-MeDHI) as the sole product.

DISCUSSION

The direct investigation of dopachrome-rearranging enzymes has been an arduous task, owing to marked difficulties in obtaining highly purified samples. In the case of DT, such difficulties account for significant differences in the properties of enzyme preparations from various sources, and even from the same source but from different laboratories. Most of what is presently known about DT has derived from the identification of the gene encoding TRP-2 (Tsukamoto et al., 1992a). The open reading frame of this gene encodes a protein with a molecular size of about 75–80 kDa, which shares significant sequence similarity (40%) with tyrosinase, including a perfect conservation of the critical domains, such as putative copper- and iron-binding regions.

Similar isolation problems prevented a detailed characterization of the dopachrome conversion factor from *Manduca*. The purified enzyme fraction exhibited two closely migrating bands on polyacrylamide gels which could be revealed by prolonged and careful staining with silver (Sugumaran, 1992).

In spite of the apparent advantage of dealing with cephalopod ink, which contains a relatively simple protein pattern, attempts to separate the *Sepia* enzyme from tyrosinase were likewise defeated. It is possible that the dopachrome-rearranging enzyme and tyrosinase occur as a tight complex, as suggested by Pawelek (1991). This, however, is difficult to assess from the results of the present study.

With respect to the catalytic properties, the *Sepia* enzyme is apparently similar to the *Manduca* enzyme. Both exhibit high stereospecificity for L-dopachrome, catalysing specifically the formation of DHI, can promote the rearrangement of α-methyl-L-dopachrome and are devoid of an effect on dopaminochrome. A major difference is, however, observed using as a substrate L-dopachrome methyl ester, in which decarboxylation is precluded. While the *Manduca* enzyme is active on this aminochrome (Sugumaran and Semensi, 1991), the *Sepia* enzyme is not, indicating that it can only act on those substrates which bear a free carboxy group. In any case, a more detailed comparison of these enzymes appears highly desirable.

The mode of action of the *Sepia* enzyme is intriguing. In the case of DT, the marked analogy of the catalytic effects with those of some transition-metal cations of biological relevance, such as Cu^{2+}, Zn^{2+} and Fe^{3+} (Leonard et al., 1988; Palumbo et al., 1987, 1991; Jara et al., 1990), which can likewise promote the non-decarboxylative rearrangement of dopachrome to DHICA, suggests that a metal ion, most probably iron, is responsible for the catalytic activity of the enzyme (Chakraborty et al., 1992). In our case a similar conclusion is not so straightforward.

Somewhat relevant is also the observation that DT is inhibited by carboxylated indole derivatives and iron chelators, such as tryptophan, indole-3-propionic acid, DHICA, 2,2′-dipyridyl, and 1,10-phenanthroline, but not by the tyrosinase inhibitors tropolone and mimosine (Aroca et al., 1991; Chakraborty et al., 1992), whereas the opposite applies to the *Sepia* enzyme. Neither DT nor the *Sepia* enzyme are, however, inhibited by phenylthiourea, a most effective tyrosinase inhibitor (Chakraborty et al., 1992). This suggests that the *Sepia* enzyme shares certain similarities with tyrosinase, but would rule out the possibility that tyrosinase itself is responsible for the dopachrome-rearranging activity, unless a different specific catalytic site is involved.

The observed ability of the *Sepia* enzyme to catalyse the rearrangement of dopachrome to DHI has important implications in relation to the structure of sepiomelanin, traditionally regarded as a model of mammalian melanin (Szabo, 1965). On the basis of analytical and degradative experiments, it was suggested that these pigments are heterogeneous polymers consisting mainly of DHI and DHICA in comparable proportions (Ito, 1986). However, while the incorporation of DHICA in mammalian melanin has recently been confirmed by a radiolabelling study on B16 melanoma tumours growing in mice (Tsukamoto et al., 1992b), the presence of this indole in sepiomelanin has never been substantiated by direct experimental evidence. In fact, a significant participation of DHICA in the biosynthesis of sepiomelanin would be difficult to reconcile with the catalytic properties of the dopachrome-rearranging enzyme reported in the present study, and until new insight is gained into the structure of the pigment, its validity as a model of mammalian melanin appears questionable.

As a concluding remark, a comment is in order regarding the nomenclature of the *Sepia* enzyme. On the basis of the reaction catalysed, this enzyme could be classified as a decarboxylase. There are grounds to believe, however, that the conversion of dopachrome into DHI is not a simple process but involves a sequence of steps, including tautomerization (Sugumaran et al., 1990; Costantini et al., 1991). Given this and the present lack of information about the structure and mode of action of the *Sepia* enzyme, we concur with Sugumaran (1992) that no specific name should be used, but only an operational definition.

This work was supported in part by grants from MURST (Rome) and CNR (Rome). We thank Miss Silvana Corsani for skillful technical assistance.

REFERENCES

Aroca, P., Garcia-Borron, J. C., Solano, F. and Lozano, J. A. (1990) Biochim. Biophys. Acta **1035**, 266–275

Aroca, P., Solano, F., Garcia-Borron, J. C. and Lozano, J. A. (1991) Biochem. J. **277**, 393–397

Aso, Y., Imamura, Y. and Yamasaki, N. (1989) Insect Biochem. **19**, 401–407

Barber, J. I., Townsend, D., Olds, D. P. and King, R. A. (1984) J. Invest. Dermatol. **83**, 145–149
Benigni, J. D. and Minnis, R. L. (1962) J. Heterocyclic Chem. **2**, 387–392
Chakraborty, A. K., Orlow, S. J. and Pawelek, J. M. (1992) FEBS Lett. **302**, 126–128
Costantini, C., Crescenzi, O. and Prota, G. (1991) Tetrahedron Lett. **32**, 3849–3850
Halaban, R. and Moellman, G. (1992) Pigment Cell Res. Suppl. **2**, 67–78
Hearing, V. J. and Tsukamoto, K. (1991) FASEB J. **5**, 2902–2909
Ito, S. (1986) Biochim. Biophys. Acta **883**, 155–161
Jackson, I. J., Chambers, D. M., Tsukamoto, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Hearing, V. (1992a) EMBO J. **11**, 527–535
Jackson, I. J., Budd, P. S., Chambers, D. M., Davidson, D., Hearing, V., Johnson, R., Steel, K. and Tsukamoto, K. (1992b) (Abstr. Annu. Meet. Panam. Soc. Pigment Cell Res. 4th) Pigment Cell Res. **5**, 86–87
Jara, J. R., Solano, F., Garcia-Borron, J. C., Aroca, P. and Lozano, J. A. (1990) Biochim. Biophys. Acta **1035**, 276–285
Kahn, V. and Andrawis, A. (1985) Phytochemistry **24**, 905–908
Korner, A. and Pawelek, J. (1980) J. Invest. Dermatol. **75**, 192–195
Kwon, B. S. (1993) J. Invest. Dermatol. **100**, 134S–140S
Kwon, B. S., Halaban, R. and Chintamaneni, S. (1989) Biochem. Biophys. Res. Commun. **161**, 252–260
Lamoreaux, M. L., Woolley, C. and Pendergast, P. (1986) Genetics **113**, 967–984
Leonard, L. J., Townsend, D. and King, R. A. (1988) Biochemistry **27**, 6156–6159
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Mason, H. S. (1948) J. Biol. Chem. **172**, 83–92
Palumbo, A., Misuraca, G., d'Ischia, M. and Prota, G. (1985) Biochem. J. **228**, 647–651
Palumbo, A., d'Ischia, M., Misuraca, G. and Prota, G. (1987) Biochim. Biophys. Acta **925**, 203–209
Palumbo, A., d'Ischia, M., Misuraca, G., Carratù, L. and Prota, G. (1990) Biochim. Biophys. Acta **1033**, 256–260
Palumbo, A., Solano, F., Misuraca, G., Aroca, P., Garcia-Borron, J. C., Lozano, J. A. and Prota, G. (1991) Biochim. Biophys. Acta **1115**, 1–5
Pawelek, J. M. (1991) Pigment Cell Res. **4**, 53–62
Prota, G. (1992) Melanins and Melanogenesis, Academic Press, San Diego
Prota, G. (1993) J. Invest. Dermatol. **100**, 156S–161S
Prota, G., Ortonne, J. P., Voulot, C., Khatchadourian, C., Nardi, G. and Palumbo, A. (1981) Comp. Biochem. Physiol. **68B**, 415–419
Shibahara, S. (1993) J. Invest. Dermatol. **100**, 146S–149S
Sobotka, H. and Austin, J. (1951) J. Am. Chem. Soc. **73**, 3077–3079
Sugumaran, M. (1992) Pigment Cell Res. **5**, 203–204
Sugumaran, M. and Semensi, V. (1991) J. Biol. Chem. **266**, 6073–6078
Sugumaran, M., Dali, H. and Semensi, V. (1990) Bioorg. Chem. **18**, 144–153
Szabo, G. (1965) in Biology of the Skin and Hair Growth (Lyne, A. G. and Short, B. F., eds.), pp. 705–726, Angus and Robertson, Sydney
Takeuchi, T. (1992) in The Pigment Cell: From the Molecular to the Clinical Level (Y. Mishima, ed.), Munksgaard, Copenhagen
Takeuchi, T., Tanaka, S. and Tanaka, M. (1993) J. Invest. Dermatol. **100**, 141S–145S
Townsend, D., Oetting, W., Polman, T. and King, R. A. (1992) Pigment Cell Res. Suppl. **2**, 32–35
Tsukamoto, K., Jackson, I. J., Urabe, K., Montague, P. and Hearing, V. J. (1992a) EMBO J. **11**, 519–526
Tsukamoto, K., Palumbo, A., d'Ischia, M., Hearing, V. J. and Prota, G. (1992b) Biochem. J. **286**, 491–495
Winder, A. J., Wittbjer, A., Rosengren, E. and Rorsman, H. (1993) J. Cell Sci. **106**, 153–166
Yamamoto, H., Takeuchi, S., Kudo, T., Sato, C. and Takeuchi, T. (1989) Jpn. J. Genet. **64**, 121–135

Received 5 August 1993/23 November 1993; accepted 13 December 1993