Dopamine in the ink defence system of *Sepia officinalis*: biosynthesis, vesicular compartmentation in mature ink gland cells, nitric oxide (NO)/cGMP-induced depletion and fate in secreted ink¹

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The biosynthesis, localization and fate of catecholamines in the ink gland of the cuttlefish *Sepia officinalis* were investigated by combined biochemical and immunohistochemical methodologies. HPLC analysis of crude ink gland extracts indicated the presence of dopa (2.18 ± 0.82 nmol/mg of protein) and DA (dopamine, 0.06 ± 0.02 nmol/mg of protein), but no detectable noradrenaline or adrenaline. DA was shown to derive from L-tyrosine, according to experiments performed by incubating intact ink glands with [L-¹⁴C]tyrosine. The biosynthetic process involves a tyrosine hydroxylase and a dopa decarboxylase pathway and is independent of tyrosinase. The tyrosine hydroxylase activity was detected under conditions of tyrosinase suppression in the cytosolic fraction, but not in the melanosomal fraction, of ink gland extracts, and the presence of the enzyme was confirmed by Western-blot analysis. Dopa and DA were found to be released from the ink glands by processes controlled through the NMDA-nitric oxide-cGMP (where NMDA stands for *N*-methyl-D-aspartate) signalling pathway, as apparent from incubation experiments performed with [L-¹⁴C]tyrosine in the presence of NMDA,

diethylamine NONOate (diethylamine diazeniumdiolate), a nitric oxide donor, 8-bromo-cGMP or a guanylyl cyclase inhibitor. Immunohistochemical results coupled with electron microscopy indicated that DA was concentrated in vesicles specifically localized in the mature melanin-producing cells of the ink gland proximal to the lumen and separated from the melanin-containing melanosomes. NMDA receptor stimulation or exposure to an NO donor caused a marked loss of DA immunoreactivity in mature cells, consistent with a release process. In the lumen of the ink gland, where mature exhausted cells pour their contents, DA immunoreactivity was found to be associated with the melanin granules, due apparently to physical adsorption. Overall, these results point to DA as a marker of cell maturation in *Sepia* ink gland subject to release by the NO/cGMP signalling pathway, and disclose apparently overlooked DA–melanin interactions in secreted ink of possible relevance to the defence mechanism.

Key words: dopa, dopamine, nitric oxide, *N*-methyl-D-aspartate receptor (NMDA receptor), *Sepia officinalis*.

INTRODUCTION

The ink defence system of the cuttlefish *Sepia officinalis* depends on the activity of a highly specialized organ, the ink gland, which is responsible for the continuous production of the black insoluble melanin pigment that the animal accumulates in the ink sac and ejects on demand [1–3]. The ink gland consists of two distinct zones exhibiting different cellular and biochemical characteristics [4,5]: an inner zone consisting of immature unpigmented cells and an outer portion made up of mature pigmented cells. The immature cells possess tyrosinase, the committed enzyme in melanogenesis; however, they are not competent for pigment synthesis and acquire this ability only during maturation. The mature cells possess catalytically active tyrosinase which is capable of promoting the conversion of tyrosine into melanin within specific organelles called melanosomes. At the end of the maturation process, the heavily pigmented cells of the outer layer shed their contents into the lumen of the gland by a still poorly defined mechanism. As a result, both melanin granules and other exhausted materials are found among ink constituents [5–7].

Although tyrosine metabolism is the most peculiar biochemical feature of the ink defence system, current knowledge and appreciation of the process are incomplete and limited to its visible

outcome, i.e. melanin formation. In fact, in addition to melanin, both dopa and DA (dopamine) have been detected in squid ink [8] and different concentrations of dopa, ranging from 1 to 500 nM, have been determined in the ejected ink of *Sepia* [9], which hints at a broader significance of tyrosine metabolism in cephalopods than the mere production of melanin.

Little is also known about the regulatory mechanisms involved in tyrosine metabolism in the ink gland. In previous studies, we demonstrated that the inner immature portion of the ink gland exhibits NMDAR1 glutamate-receptor-like immunoreactivity and contains a calcium–calmodulin-dependent nitric oxide synthase [10]. Stimulation of NMDA (*N*-methyl-D-aspartate) receptors caused a marked increase in cGMP levels, activation of tyrosinase and increased melanin synthesis in the mature portion of the gland, via the NO–guanylyl cyclase interaction [11]. Whether the NMDA/NO/cGMP pathway controls other processes relating to tyrosine metabolism has remained unknown so far.

In the present study, we used biochemical and immunohistochemical methods to investigate the origin, localization and fate of catecholamines in the ink gland of *S. officinalis*. The effects of an activated NO/cGMP signalling pathway on catecholamine levels are also addressed.

Abbreviations used: BH₄, (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride; 8-Br-cGMP, 8-bromo-cGMP; DA, dopamine; DEA/NO, 2-(*N,N*-diethyl-amino)-diazeneolate-2-oxide; DTT, dithiothreitol; NMDA, *N*-methyl-D-aspartate; ODQ, 1*H*-(1,2,4)oxadiazolo[4,3-*a*]quinoxaline-1-one.

¹ Dedicated to the memory of Professor Giuseppe Prota (1938–2003).

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EXPERIMENTAL

Animals

Specimens of *S. officinalis* were collected in the Bay of Naples and kept in a controlled environment for 2 days. Dissections were performed using cold anaesthesia. The ink contained in the ink sac was collected, and the ink glands were removed and used immediately.

Materials

8-Br-cGMP (8-bromo-cGMP) was from Calbiochem (Inalco, Milan, Italy). DEA/NO [2-(*N,N*-diethylamino)-diazolot-2-oxide] and BH₄ [(6*R*)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride] were obtained from Alexis Italia (Vinci, Italy). L-[U-¹⁴C]Tyrosine (456 mCi/mmol, 50 µCi/ml) and L-[3,5-³H]tyrosine (55.0 Ci/mmol, 1.0 mCi/ml) were from Amersham Italia (Milan, Italy). All other chemicals were from Sigma (Milan, Italy). EnVision+ was obtained from Dako Corporation (Carpinteria, CA, U.S.A.).

Incubation experiments with intact ink glands, and dopa and DA determination

Single ink glands were incubated at 20 °C in sea water (2 ml) with gentle agitation in the presence of 1.1 µM L-[U-¹⁴C]tyrosine. When necessary, one of the following additives was added: 1.5 mM NMDA, 1.25 mM DEA/NO or 7.5 mM 8-Br-cGMP. Before stimulation with NMDA, ink glands were preincubated for 30 min with 100 µM ODQ {1*H*-(1,2,4)oxadiazolo[4,3-*a*]quinoxaline-1-one}, when required. After incubation for appropriate intervals of time, the tissues and the incubation media were collected, immediately stored at -80 °C and then examined for catecholamine content.

Radioactive single ink glands were homogenized in 200 µl (100 mM) of ice-cold sodium acetate buffer (pH 4.5). After centrifugation at 51 000 *g* for 20 min, the supernatant was treated with perchloric acid (final concentration 0.1 M) and centrifuged at 15 000 *g* for 15 min. The supernatant was analysed for catecholamines by HPLC. When necessary, the incubation media were centrifuged at 15 000 *g* for 15 min, and the supernatants were freeze-dried, dissolved in HPLC buffer (100 µl) and examined for catecholamine content. For catecholamine determination, pools of ink glands (about 10 glands/pool) were used. The extract was prepared as described above and was treated with alumina as reported previously [12]. HPLC was performed using a Gilson model 322 instrument with a Spheroclone ODS2 column (5 µm pore size; 250 mm × 4.60 mm; Phenomenex Chemtek Analytica, Bologna, Italy). The mobile phase was 25 mM sodium phosphate buffer (pH 3), containing 5 mM octanesulphonic acid/acetonitrile (91:9, v/v). The flow rate was maintained at 1 ml/min. Detection was at 280 nm with a Gilson UV/VIS-152 detector, by electrochemical detection and by liquid-scintillation counting of each fraction. The electrochemical detection system (ESA Coulochem II) comprised a guard cell (model 5020) and a standard analytical cell (model 5011) containing dual coulometric detectors. The guard cell was set at +500 mV; the working potentials of the first and second electrodes of the analytical cell were +400 mV and -200 mV respectively. Dopa and DA were identified by co-injection with authentic samples, as well as by their electrochemical behaviour.

Assay of enzyme activities in ink gland extracts

Ink glands were homogenized in ice-cold 50 mM Tris/HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT (dithiothreitol), sup-

plemented with 10 µg/ml leupeptin, 10 µg/ml pepstatin A and 100 µg/ml PMSF. All subsequent manipulations were performed at 4 °C. After centrifugation at 700 *g* for 10 min, the pellet was re-extracted with the same buffer and centrifuged. The combined supernatants were centrifuged at 51 000 *g* for 20 min and the supernatant was used as a cytosolic fraction for enzymic assays. The melanosome-containing pellet was subjected to sucrose-density-gradient ultracentrifugation as described previously [11]. The brown melanosomal fraction at the bottom of 30 % sucrose was extensively washed with 0.1 M Tris buffer (pH 7.2) and collected by centrifugation. The pellet was resuspended in 100 µl of extraction buffer as described previously [13] and used for enzymic assays.

Tyrosinase hydroxylase activity was assayed essentially as described previously [14]. Briefly, each assay (50 µl) contained 0.5 µCi L-[3,5-³H]tyrosine, 25 µM L-tyrosine, C-100 catalase (4600 units), 5 mM DTT, 0.5 mM BH₄ and 50 mM Mes buffer (2-[*N*-morpholino]ethanesulphonic acid, pH 6.1). Reactions were initiated after the addition of suitable amounts of cytosolic (0.4 µg of protein) and melanosomal (465 µg of protein) fractions and were incubated at 37 °C for different times. After incubation, 0.95 ml of 2.5 % trichloroacetic acid containing activated charcoal (65 mg/ml) and Celite 545 (65 mg/ml) was added to the sample and agitated continuously for 1 h at room temperature (25 °C). After centrifugation at 7400 *g* for 15 min, 500 µl of supernatant was examined by liquid-scintillation counting.

Dopa decarboxylase activity was determined by a modification of a reported procedure [15]. Briefly, 1.36 mg of cytosolic fraction was incubated in 0.03 M sodium phosphate buffer (pH 6.9), at 37 °C with 0.25 µCi of L-[U-¹⁴C]tyrosine, 1 mM L-tyrosine, 5 mM DTT, 0.5 mM BH₄, 0.1 mM pargyline and 0.2 mM EDTA. The reaction was started by the addition of 0.01 mM pyridoxal 5'-phosphate. After 1 h, aliquots were withdrawn, treated with perchloric acid (final concentration 0.1 M), centrifuged at 15 000 *g* for 15 min and analysed by HPLC. The mobile phase was 25 mM sodium phosphate buffer (pH 3), containing 5 mM octanesulphonic acid/acetonitrile (22:3, v/v). Detection was performed at 280 nm and by liquid-scintillation counting of each fraction. The elution times for dopa, tyrosine, DA and tyramine were 8.1, 11.5, 27 and 42 min respectively.

Melanin-binding studies

The ink was centrifuged at 51 000 *g* for 1 h and the supernatant was used as melanin-free ink. The melanin precipitate, extensively washed with sea water to remove tyrosinase activity [6], was resuspended in sea water. Convenient aliquots of ink, tyrosinase-free melanin suspension or melanin-free ink were allowed to react at 23 °C with 1 mM solution of DA in sea water. At different times, aliquots were withdrawn, filtered through 0.22 µm filters when necessary and examined by recording UV/visible spectra and by HPLC analysis. HPLC was performed as described above with detection at 280 nm. The amount of melanin used in each experiment was measured by weighing convenient aliquots of tyrosinase-free melanin suspension or whole ink after drying on silica gel.

Western-blot analysis

Ink gland extracts were prepared as reported previously [10]. Proteins were solubilized in Laemmli's sample buffer and separated by SDS/PAGE (7.5 % gel) and transferred on to Hybond enhanced chemiluminescence nitrocellulose (Amersham). After blocking with non-fat milk in PBS, the filters were incubated for 1 h at room temperature with rabbit anti-tyrosine hydroxylase

polyclonal antibody (1:1000; Chemicon AB 151). After washing, the bound antibody was visualized by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions. In control experiments, primary antibody was omitted. Molecular masses were determined by comparison with rainbow coloured protein molecular-mass standards (Amersham). Protein content was determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Milan, Italy) with BSA as a standard.

Immunohistochemical analyses

For immunohistochemical staining, intact ink glands, incubated for 1 h in sea water (2 ml) in the absence or presence of 1.5 mM NMDA, were pre-washed for 5 min in 50 mM cacodylate buffer (pH 7.4), containing 1 % MBS (sodium metabisulphite). The glands were then fixed overnight by immersion in a solution of 5 % glutaraldehyde in 100 mM cacodylate buffer and 1 % MBS (pH 7.4) and embedded in sucrose by gradually increasing its concentration up to 30 %. Dried cryostat sections collected on slides pretreated with Biobond (British Biocell International, Cardiff, U.K.) were incubated with 10 % normal goat serum for 30 min at room temperature in a moist humid chamber to reduce the background staining. After a 30 min treatment with 3 % H_2O_2 to block the endogenous peroxidases, the sections were incubated overnight with rabbit polyclonal antiserum raised against DA (1:1000; Chemicon AB122S). The sections were subsequently incubated in EnVision+ (anti-rabbit immunoglobulins conjugated to peroxidase-labelled dextran polymer) for 30 min at room temperature. The labelled sections were visualized by using diaminobenzidine as chromogen. Sections were dehydrated in ethanol series, cleared in xylene and mounted by using Biomount (British Biocell International). Sections were viewed and photographed through Nikon Eclipse E800. Images of ink gland sections were analysed by using a CCD video camera, and screen measurements were made using LUCIA analysis software image (Laboratory Imaging, Nikon, U.K.). For control of non-specific DA immunoreactivity, experiments were performed by omitting the primary antibody. For ultrastructural identification of catecholamine vesicles, intact ink glands were treated as reported for other systems [16]. Immunolocalization of DA in the ejected ink at ultrastructural level was performed as described previously [5].

Statistical analysis

Results were compared using two-tailed Student's *t* test. Reported values are means $\pm$ S.D.

RESULTS

Catecholamines in the ink gland of *S. officinalis*

HPLC analysis of crude extracts from pools of freshly collected *Sepia* ink glands indicated the presence of dopa and DA (2.18 ± 0.82 and 0.06 ± 0.02 nmol/mg of protein respectively as determined for three pools of ten glands each), but no detectable noradrenaline or adrenaline. The identities of dopa and DA were determined by spiking the extracts with authentic samples and by careful comparison of their electrochemical responses.

Incubation of intact ink glands in sea water in the presence of [^{14}C]tyrosine resulted, after 30 min, in a well-detectable incorporation of radioactivity at the level of dopa (up to 5.0 c.p.m./ μg of protein) and DA (up to 1.07 c.p.m./ μg of protein) in the glands (Figure 1).

Notably, after 4 h, small amounts of radiolabelled dopa and DA (see below) were also detected in the incubation waters, indicating

Figure 1 Biosynthesis of catecholamines in the ink gland of *S. officinalis*

Single intact ink glands were incubated with gentle agitation at 20 °C in sea water containing 1.1 μM [^{14}C]tyrosine. Glands were collected at fixed intervals of time, homogenized and deproteinized as described in the Experimental section. Each sample was examined by HPLC and the fractions were monitored spectrophotometrically at 280 nm, by liquid-scintillation counting and electrochemical detection. Products were identified by comparison/co-injection with authentic standards. Results are expressed as c.p.m./ μg of protein determined before deproteinization of the extract. Results represent means $\pm$ S.D. for $n = 5$. $\diamond$, DOPA; $\square$, DA.

Figure 2 Tyrosine hydroxylase activity in cytosolic and melanosomal fractions of the ink gland extract

Cytosolic and melanosomal fractions, prepared as described in the Experimental section, were incubated at 37 °C for 30 min in the presence of cofactors. Results are means $\pm$ S.D. for five separate experiments. Statistical significance: ** $P < 0.01$. Open bars, in the presence of 0.5 mM BH_4 and 5 mM DTT; hatched bars, in the absence of BH_4 and DTT.

that the catecholamines synthesized in the glands were partially released from the organs.

On the basis of these results, the enzyme activities involved in DA formation in the ink gland were investigated. Tyrosine hydroxylase was assayed by a procedure based on the determination of $^3\text{H}_2\text{O}$ released from [3,5- ^3H]tyrosine in the presence of BH_4 and DTT as essential cofactors for enzyme activity [14]. A well-detectable tyrosine hydroxylase activity depending on BH_4 and DTT was observed in the cytosolic fraction but not in the melanosomal fraction (Figure 2). In control experiments, BH_4 and DTT were found to suppress *Sepia* tyrosinase activity completely (results not shown), as reported for the human enzyme [14].

In additional experiments, [^{14}C]tyrosine was incubated with the cytosolic fraction of ink gland extracts in the presence of BH_4 , DTT and pargyline as monoamine oxidase inhibitor. HPLC analysis after 1 h incubation showed the formation of labelled dopa (6.7 ± 2.1 pmol of dopa/mg of protein) and DA (1.3 ± 0.8 pmol of DA/mg of protein), confirming the presence of tyrosine hydroxylase and demonstrating dopa decarboxylase activity [15]. Notably, no detectable formation of labelled tyramine was observed.

Presence of tyrosine hydroxylase was also demonstrated by SDS/PAGE analysis of extracts of *Sepia* ink glands, revealing an intense band at 60 kDa, immunoreactive to antibodies against

Figure 3 Western-blot analysis of *Sepia* ink gland extract

A solubilized tissue sample (75 μ g) was transferred on to a nitrocellulose membrane after SDS/PAGE and immunostained with antibodies raised against tyrosine hydroxylase (1:1000). Numbers on the left indicate the positions of molecular-mass standards (kDa).

tyrosine hydroxylase (Figure 3). Omission of the primary antibody eliminated the immunoreactivity (results not shown).

Effects of the NMDA/NO/cGMP signalling pathway on dopa and DA levels in the ink glands

NMDA stimulation of intact ink glands exposed to [14 C]tyrosine resulted in a marked decrease in the levels of radioactive dopa and DA with respect to unstimulated ink glands (Figure 4A). The effect was time-dependent and was clearly apparent after 1 h incubation. A similar time-dependent trend was observed when the ink glands were exposed to DEA/NO, a widely used NO donor (Figure 4B).

HPLC analysis of the sea water media of ink glands incubated for 4 h with NMDA or DEA/NO revealed an increase in the levels of radiolabelled dopa (1560 ± 200 and 1990 ± 150 c.p.m./ng of protein of the corresponding ink gland, respectively; $P < 0.01$ in both cases) with respect to controls (560 ± 150 c.p.m./ng of protein of the corresponding ink gland). Similarly, incubation with NMDA and DEA/NO caused an increase in DA radioactivity in the incubation waters (260 ± 30 and 320 ± 30 c.p.m./ng of protein of the corresponding ink gland, respectively; $P < 0.1$, $P < 0.01$ with respect to controls (205 ± 30 c.p.m./ng of protein of the corresponding ink gland). The latter results were poorly reproducible due to experimental difficulties caused by the presence of melanin in the incubation media affecting quantitative determinations and the variability inherent to experiments with whole organs.

8-Br-cGMP, a membrane-permeant, phosphodiesterase-resistant active analogue of cGMP, mimicked NMDA and DEA/NO in causing nearly a 50% decrease in the radioactivity levels of dopa and DA in the ink glands after 4 h exposure. Conversely, ODQ, a specific inhibitor of guanylyl cyclase, decreased the extent of the NMDA-induced depletion of dopa and DA in the ink gland by approx. 50%.

DA immunohistochemistry in the ink gland

Figures 5(A) and 5(B) show the results of immunolocalization experiments on ink gland sections using antibodies raised against

Figure 4 Effects of NMDA and DEA/NO on the levels of newly synthesized catecholamines in the ink gland of *S. officinalis*

Single intact ink glands were incubated with [14 C]tyrosine as detailed in Figure 1 in the presence of 1.5 mM NMDA (A) or 1.25 mM DEA/NO (B). Glands were collected at fixed intervals of time, homogenized, deproteinized and analysed by HPLC for catecholamine levels as described previously. Data were determined as c.p.m./ μ g of protein, and the results are reported in terms of percentage values (means $\pm$ S.D., $n = 5$) with respect to the corresponding controls in the absence of NMDA or DEA/NO. Statistical significance: * $P < 0.05$, ** $P < 0.01$ compared with the corresponding controls. Black bar, DOPA; open bar, DA.

DA. A strong immunoreactivity was localized in the mature melanized cells of the outer cell layer proximal to the lumen and in the ink. No immunopositive staining was observed in the inner glandular cell layer. Staining was completely abolished when the primary antibody was omitted. Immunogold experiments revealed distinct gold particles decorating melanin granules secreted into the lumen (inset to Figure 5B).

Interestingly, a marked loss of DA-like immunoreactivity was observed after the exposure of ink glands for 1 h to NMDA (Figure 5C) or NO donors (not shown), consistent with a NO-induced release of the catecholamine from the organs.

Subcellular localization of DA in mature ink gland cells

For subcellular localization of DA, ink gland sections were analysed at an ultrastructural level. Electron-dense vesicles were present in the cytoplasm of mature melanized cells proximal to the lumen (Figure 6). No vesicles were present in the ejected ink. No exocytotic profiles were observed. The vesicles were irregular in size and approximately spherical. They showed a bounding membrane and a mean diameter of about 50 nm. The cores of the vesicles showed variable electron density.

DA in secreted ink

The results of immunolocalization experiments at the ultrastructural level (inset to Figure 5B) demonstrated that DA in secreted

Figure 5 DA immunohistochemistry in the ink gland

(A, B) Section from tissue slice incubated for 1 h in sea water. (A) Immunoreactivity is localized in the outer layer (arrows). A white asterisk indicates melanin. Magnification $\times 260$. (B) A cloudy immunoreactivity is present at the level of ejected ink (ei) (arrows). lu, lumen. Magnification $\times 850$. The inset shows immunogold localization of DA in the ejected ink. Gold particles can be seen on melanin granules (arrows). Bar: 50 nm. (C) Section from tissue slice incubated for 1 h in sea water containing NMDA. No immunopositivity was observed. Magnification $\times 260$.

ink is physically absorbed on to the melanin granules. Accordingly, in a final set of experiments, the interaction of DA with the ink was investigated. HPLC analysis (Figure 7) showed that

Figure 6 Ultrastructural analysis of *Sepia* ink gland

Identification of catecholamine-containing vesicles. High magnification of electron-dense vesicles of about 50 nm (arrows) in the cytoplasm of mature melanized cells. The cores of the vesicles show a variability of electron density. Bar: 0.1 μ m.

Figure 7 Fate of DA in secreted ink

Aliquots of ink and tyrosinase-free melanin suspension containing the same amount of melanin (16 mg) or the corresponding melanin-free ink were added to a 1 mM solution of DA in sea water. At zero time (open bars), 5 min (hatched bars), 15 min (black bars) and 30 min (cross-hatched bars) aliquots were withdrawn, filtered through 0.22 μ m filters when necessary and DA content was measured by HPLC analysis as described in the Experimental section. The results are reported in terms of percentage values (means $\pm$ S.D., $n = 5$) with respect to the corresponding value at zero time.

the addition of a whole ink suspension to a solution of DA in sea water caused a rapid decrease in the catecholamine concentration. A similar effect was observed when freshly collected melanin, previously washed with sea water to remove tyrosinase and other colourless ink constituents, was added to the DA solution. In both cases, UV/visible and HPLC monitoring did not indicate the formation of DA oxidation products, e.g. the visible dopaminochrome or 5,6-dihydroxyindole [17]. With the melanin-free ink, DA decay was much slower and a concomitant oxidation of DA was revealed by UV/visible spectrophotometry. Control experiments showed that DA was sufficiently stable in sea water under the same experimental conditions, average consumption being $< 5\%$ after 30 min.

DISCUSSION

Sepia ink gland cells have traditionally been regarded as mere melanin-producing cells [1,3–5]. This view stemmed largely from the deep-rooted concept of the ink as a visual screen, and has

profoundly affected the researchers' attitude towards this intriguing defence system. As a result, several important biochemical and ultrastructural features of the ink gland appear to have been overlooked. The present demonstration that in the mature ink gland cells of *S. officinalis* part of the tyrosine pool is converted into DA in specific compartments distinct from melanosomes discloses new important aspects of the biochemistry of this organ of potential relevance to the complex mechanisms underlying the ink defence system.

The specific localization of DA in the outer melanin-producing cells, but not in the inner immature ones, would point to DA as a marker of cell maturation in the ink gland, in addition to active tyrosinase and melanin. In this organ, DA might well play an active role in the mechanisms of cell maturation, consistent with the reported ability of catecholamines to regulate morphogenic activities such as proliferation, differentiation and metamorphosis, in addition to functioning as neurotransmitters and hormones [18–20].

The ultrastructural analysis of mature ink gland cells reported herein, together with previous studies on the localization of melanogenic enzymes in *Sepia* ink gland [5], showed that DA and melanin are present in the cytoplasm of mature cells in separate compartments. Whereas melanin is confined to the melanosomes, DA appears to be segregated within electron-dense vesicles resembling the catecholamine-containing vesicles reported in other systems [16,21]. This would entail that DA is not primarily involved in melanin synthesis, consistent with the results of previous chemical studies [22]. Only when the maturation process is complete and the outer cells shed their contents into the lumen, DA-containing vesicles are disrupted and the catecholamine becomes available to interact with the pigment. The possibility that the observed immunoreactivity on melanin granules could be due to cross-reactivity of the anti-DA antibodies with uncyclized units in the melanin polymer is incompatible with the following arguments. First, cross-reactivity of the antibody to dopa, the prototypic uncyclized unit in the melanin polymer, is ruled out by control experiments provided by the antibody manufacturer. Secondly, immunohistochemical experiments on gland tissues incubated with NMDA (see Figure 5C) showed a marked loss of immunoreactivity of pigmented cells with respect to controls, indicating that melanin, by itself, does not give rise to cross-reactivity with the antibodies. Finally, the physical adsorption mechanism provides a convincing explanation to the marked decrease in free DA concentration observed after the addition of melanin to catecholamine solutions in sea water. The proposed physical adsorption mechanism, although previously unrecognized, would not be unexpected, considering the extensive body of literature documenting the binding properties of melanins towards organic materials and cationic species [23].

Biosynthetically, DA appears to arise from the hydroxylation of tyrosine to dopa promoted by tyrosine hydroxylase and subsequent dopa decarboxylation by the action of dopa decarboxylase (Scheme 1). It is worth noting that a tyrosinase-dependent, tyrosine hydroxylase-independent mechanism of DA synthesis has recently been reported [24] as a major determinant of peripheral DA production during postnatal development in pigmented mice. During this period, DA may be formed by decarboxylation of L-dopa produced during melanin synthesis, as a result of redox cycling of dopaquinone with leucodopachrome (cyclo-dopa), or alternatively by decarboxylation of tyrosine to tyramine and subsequent hydroxylation by tyrosinase. With aging, tyrosinase-dependent DA synthesis in mice was reported to decrease, due to a developmental switch to production of melanin [24]. In view of this report, the possibility that tyrosinase provided a vicarious tyrosine hydroxylase activity leading to DA

Scheme 1 Tyrosine metabolism in the ink gland

in the mature ink gland cells was considered. The suppressive effect of DTT on tyrosinase activity, however, ensured that under such conditions virtually all of the DA derived from labelled L-tyrosine was produced via the tyrosine hydroxylase activity. Our failure to detect labelled tyramine, furthermore, would rule out a major contribution of tyrosine hydroxylase-independent pathways of DA synthesis triggered by tyrosine decarboxylation [24]. Thus, it can be concluded that in *Sepia* ink glands, tyrosine partitions between the tyrosinase-dependent melanogenic pathway in the melanosomes and the tyrosine hydroxylase-dependent DA-forming route in vesicles (Scheme 1), with no apparent interplay.

The demonstration of two branches of tyrosine metabolism (i.e. melanogenesis and DA synthesis) in mature ink gland cells is of interest also in relation to the recent report of tyrosine hydroxylase and tyrosinase in melanosomes of human epidermal melanocytes, in which the two enzymes have been proposed to act in concert to promote pigmentation [14]. In the melanocytes, tyrosinase and tyrosine hydroxylase would show side-by-side melanosomal membrane association and would also share the same biosynthetic origin from the *trans*-Golgi network. The results obtained from within the mature ink gland cells, on the other hand, suggest a quite different situation in which the enzymes are confined to different compartments and are deputed to the control of separate biochemical pathways. It is of relevance to notice that no tyrosine hydroxylase activity has been detected in the melanosomal fraction in *Sepia*. Elucidation of these pathways may lead to improved understanding of physiological and pathological processes involving aberrant tyrosine metabolism and DA synthesis in settings of pigmentary alterations, e.g. vitiligo [25–27].

The finding that stimulation of the NO/cGMP signalling pathway causes a depletion of DA and dopa in the ink gland with an increase of the label in the incubation waters hints at a new possible role of NO in the control of ink secretion from mature cells, in analogy with similar processes occurring in other secretory glands [28,29]. Current uncertainties about the detailed mechanism of ink secretion prevent us from drawing conclusions as to how NO promotes DA and dopa release from mature cells via cGMP.

Free dopa and DA have already been detected among the low-molecular-mass constituents of the ejected ink of squid [8] and cuttlefish [9], but no evidence has so far been reported about their occurrence and localization in ink gland cells in *Sepia*.

The finding that DA in secreted ink is adsorbed on to melanin granules may yield new interesting clues about the mechanism of action of the ink and its role in the behaviour of cephalopod intra-/inter-specific relationships, i.e. defence and schooling behaviour. Scattered observations in the literature suggest that the actual function of the ink does not relate to its dark appearance, but rather to the presence of colourless bioactive constituents [2,3]. According to an early suggestion, the occurrence of large amounts of tyrosinase in the ejected ink would ensure efficient conversion of catechols into toxic quinones acting as deterrent for the predator [6]. Along this line of thought, later studies indicated that dopa and DA are active on squid olfactory neurons [30]. On the basis of the results of the present study, it may be speculated that melanin granules in the secreted ink serve as a sink/carrier for DA, preventing excessive dilution after ejection and ensuring efficient interactions with target organs in DA-mediated inter- and intra-species communication.

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