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Egg Capsule Catechol Oxidase from the Little Skate *Raja erinacea* Mitchill, 1825*

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Abstract. A phenoloxidase was demonstrated in extracts of egg capsules tanning *in utero* and of nidamental glands from spawning little skate, *Raja erinacea*. The enzyme was identified as a catechol oxidase based on its ability to oxidize the *ortho*-diphenols pyrocatechol, 4-methylcatechol, 3,4-dihydroxyphenylalanine, 3-hydroxytyramine and *N*-acetyldopamine to their corresponding *ortho*-quinones and its relative inactivity against monophenols. 4-methylcatechol was oxidized at the greatest rate, while 3,4-dihydroxyphenylalanine, 3-hydroxytyramine and *N*-acetyldopamine were oxidized at slower rates. The nidamental gland enzyme was inhibited by cyanide, nitrogen, and diethyldithiocarbamate. Oxidase activity in crude extracts from nidamental glands was enhanced by addition of α-chymotrypsin, suggesting that the enzyme is produced in a latent form. Ammonium sulfate fractionation of nidamental gland and capsule extracts resulted in a fifteen-fold purification of the enzyme. This partially purified catechol oxidase from the nidamental gland exhibited optimal rates of oxidation at 0.5 *M* NaCl and pH 7.0. The enzyme, however, showed a wide tolerance for elevated salinity and alkaline pH. These observations indicate that the oxidase acts principally *in utero,* but may remain active in seawater following oviposition of the capsule. This enzyme plays a pivotal role during the formation of skate egg capsules by catalyzing the oxidation of capsular catechols to highly reactive quinones forming dark pigments which tan the capsular matrix.

Introduction

Oviparous elasmobranchs encapsulate eggs in curiously shaped, leathery capsules produced by specialized nidamental glands in the upper oviduct (for review of the structure and composition of these capsules see Hunt, 1985). These glands are highly developed during spawning and in some species are the predominant organs in the reproductive tract. The early structural studies of Perrevex (1884), Henneguy (1893), Borcea (1904, 1905), and Widakowich (1906) established that the nidamental glands of *Scyliorhinus canicula* and several other oviparous species have distinct glandular regions each with an extensive tubular system leading to lamellae at the lumenal surface. Typically, three regions were discriminated: an albumen-secreting zone, a mucous-secreting zone, and a shell-secreting zone. The tubules in the shell-secreting zone were bordered by epithelial cells which contained abundant cytoplasmic granules filled with the precursors of capsules. During capsule formation, these granules are secreted from the epithelial cell into the lumen where they coalesce and then are transported to the lamellae by ciliated tubule cells (Filhol and Garrault, 1938). Borcea (1905) and Widakowich (1906) showed how these lamellae mould the newly secreted capsular material and produce the layered organization of the capsular wall. This basic structure appears common to nidamental glands from both oviparous sharks and skates, having now been described in *Scyliorhinus canicula* (Perrevex, 1884; Henneguy, 1893; Borcea, 1904, 1905; Widakowich, 1906; Filhol and Garrault, 1938; Metten, 1939; Threadgold, 1957; Krishnan, 1959; Rusaouën, 1978), *Chiloscyllium griseum* (Nalini, 1940), *Raja batis,* and *Raja miraletus* (Filhol and Garrault, 1938). Although reduced in overall complexity and size, the nidamental glands of ovoviviparous and viviparous species also show similar tubular organization and lamellar systems (Borcea, 1905; Filhol and Garrault, 1938; Nalini, 1940; Prasad, 1945a, 1945b, 1948).

Rusaouën (1978) and Rusaouën *et al.* (1976) provided

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* Portions of this work have appeared in abstract form; see Koob & Cox, 1984, 1985, and 1986a.

ultrastructural and histochemical evidence that six zones of secretory activity containing five types of secretory granules could be distinguished in the nidamental glands of *Scyliorhinus canicula*. Histochemical tests identified neutral and sulfated mucopolysaccharides, sulfated glycoproteins rich in tyrosine, a fibrillar collagenous protein, sulfhydryl groups, indole radicals, peroxidase, and phenoloxidase activities, each localized in granules of specific regions and cell types within the glands. In the shell secreting zone alone, Rusaouën (1978) found all of these components except the mucopolysaccharides. Her studies provide convincing evidence that the nidamental gland is an extremely complex organ which synthesizes a variety of secretory products and that capsule formation and composition are equally complex.

Formation of skate egg capsules begins in the nidamental gland with the secretion and assembly of capsular precursors. These materials are white when assembled but then gradually develop color with time *in utero*, eventually producing the deep greenish brown characteristic of skate capsules at oviposition. In *Raja erinacea*, the tanning of capsules *in utero* is coincident with the introduction of catechols into the capsular matrix (Koob and Cox, 1986b). An enzymic activity able to oxidize catechols to quinones has been demonstrated histochemically in tanning capsules and nidamental glands from several oviparous elasmobranchs. Brown (1955) reported that sections of newly formed *Raja* egg capsules turned brown upon incubation with tyrosine and that this reaction could be blocked by potassium cyanide. She believed that these results demonstrated a polyphenol oxidase which would oxidize the polyphenol present in the capsule to quinone which, in turn, would tan the capsule. A polyphenoloxidase was demonstrated histochemically in shell glands of *Scyliorhinus canicula* by incubating sections of fixed glands with catechol (Threadgold, 1957). Krishnan (1959) showed that both capsular material and sections of frozen glands from *Chiloscyllium griseum* oxidized catechol and that the capsule had chemical properties like other quinone tanned matrices. He suggested that capsule formation involved a form of quinone autotanning (*sensu* Smyth, 1954) catalyzed by a phenoloxidase. In *Scyliorhinus canicula* this enzyme is localized both to a narrow zone in the upper region of the nidamental gland and to a broad band in the caudal region (Rusaouën, 1978). Further information regarding the nature of this oxidase activity is lacking.

Therefore we set out to characterize the biochemical properties of the oxidase involved in forming egg capsules of the little skate, *Raja erinacea*. We were especially interested in defining the substrate specificity of the oxidative activity and in determining the sensitivity of this activity to inhibitors, salinity, pH, and urea to gain insight into the conditions within the tanning capsular matrix.

Materials and Methods

Selection of animals

Females of *Raja erinacea* were selected from otter trawl catches on the basis of ovarian size and color as viewed through the translucent ventral body wall. Bumpus (1898) showed that ripe females can be discriminated in this way. We found that females so selected will produce egg capsules during short term captivity (Koob *et al.*, 1986). Females landed with capsules *in utero* were also selected. Skates were maintained in 2400 l aquaria supplied with fresh circulating seawater and were fed Maine Gulf shrimp. Every twelve hours females were palpated for egg capsules in the uterus. Only females that produced eggs were used for collection of nidamental glands.

Egg capsule preparation

A female which had just completed secretion of egg capsules was sacrificed and the oviducts containing newly formed capsules were excised *in toto*. Later examination revealed that the egg capsules were fully formed but untanned at the anterior, more recently secreted end. The oviducts were ligated at the cervix and just cephalad to the nidamental gland to isolate the tanning capsule within the uterine portion of the oviduct. Ten ml of 1.0 *M* NaCl, 0.05 *M* NaH_2PO_4, pH 7.5 chilled to 4°C were introduced into each uterine lumen with a syringe *via* the cervical canal. After manipulating the buffer to thoroughly wash the uterine contents, it was collected through the opened upper oviduct. This uterine flush contained much particulate which was removed by centrifugation at 3,000 rpm and 4°C for 10 minutes. The capsules were then removed from the oviduct and placed into 60 ml of the same salt buffer at 4°C with occasional stirring for 15 minutes. This capsular wash was centrifuged as above to remove particulates. The attachment fibers from these capsules were then removed from the lateral seams and homogenized on ice in 30 ml of the 1.0 *M* NaCl buffer using a glass homogenizer. The homogenate was centrifuged at 37,000 × *g* for 15 minutes at 4°C. The supernatants from the uterine flush, capsular wash, and attachment fiber extract were assayed directly for oxidase activity.

A second, partially tanned capsule was removed from the uterus and extracted directly with 1.0 *M* NaCl, 0.05 *M* NaH_2PO_4, pH 7.0 by homogenization with a polytron (Brinkmann Instruments Inc., Westbury, New York). Following centrifugation at 37,000 × *g* and 4°C for 30

minutes, the extract was fractionated by differential ammonium sulfate precipitation as described below.

Nidamental gland preparation

Nidamental glands from spawning females were excised from the oviduct, minced over ice and disrupted with a glass homogenizer in 1.0 *M* NaCl, 0.05 *M* NaH_2PO_4, pH 7.0. The homogenate, which appeared gelatinous and slightly pink, was centrifuged at 37,000 × *g* and 4°C for thirty minutes. The pinkish supernatant was collected and analyzed directly for oxidase activity. This 1.0 *M* NaCl extract was subsequently fractionated by sequential precipitation at 5, 10, 20, 30, and 40% ammonium sulfate at neutral pH. Precipitates were collected by centrifugation at 4°C and 37,000 × *g* for thirty minutes, and redissolved in 1.0 *M* NaCl, 0.05 *M* NaH_2PO_4, pH 7.0. Not all the precipitate formed at low ammonium sulfate concentrations dissolved in the buffer, therefore it was necessary to clarify these solutions by centrifuging at 25,000 × *g* and 4°C for 15 minutes. Protein determinations were performed on diluted aliquots of the original extract and on the redissolved ammonium sulfate fractions (Lowry *et al.*, 1951).

Enzyme assay

Oxidase activity was measured in the various enzyme preparations by incubating diluted aliquots with 1 m*M* substrate in 0.5 *M* NaCl, 0.025 *M* NaH_2PO_4, pH 7.5 at ambient temperature and spectrophotometrically monitoring for increases in absorbance at product specific wavelengths. Substrates generally employed for oxidase assays were 3,4-dihydroxyphenylalanine (l-dopa) or 4-methylcatechol; other substrates tested were *p*-cresol, 3-hydroxytyramine, *N*-acetyldopamine and tyrosine (all obtained from Sigma Chemical Co., St. Louis, Missouri). Extinction coefficients for the substrates were from Waite (1976). For each assay the enzyme solution and diluent were mixed in 1 ml cuvettes. The reaction was initiated by adding substrate in a 0.01 *M* HCl stock solution and mixing. The change in absorbance at product specific wavelengths was recorded for periods up to 120 minutes. All assays were performed at room temperature in a reaction volume of 1 ml. The change in absorbance in the enzyme solutions was compared to that in control incubates which contained boiled enzyme, substrate, and buffer.

The kinetic parameters K_m and V_{max} were estimated by direct linear plots (Eisenthal and Cornish-Bowden, 1974) using only the initial, briefly linear reaction velocities from assays performed as described above. This limited the usable portion of such kinetic assays to about 30 seconds.

Inhibitors of other catechol oxidases were tested for effect on skate enzyme preparations. KCN or diethyldithiocarbamate was added to a final concentration of 50 μM and incubated 15 minutes at room temperature prior to the addition of 4-methylcatechol. In all other respects the assays were performed as above. Results from these assays were used to determine the type of inhibition observed and to estimate K_i, both by means of direct linear plots (Eisenthal and Cornish-Bowden, 1974). The effect of nitrogen on oxidase activity was estimated by extensive purging of reaction solutions prior to 4-methylcatechol addition and by assaying the reactions under nitrogen.

To examine the effects of salinity, pH, and urea on oxidase activity, the desired concentrations were effected by diluting the enzyme solution with concentrated stock buffers. NaCl concentration was varied from 0.25 *M* to 1.0 *M* in 0.05 *M* NaH_2PO_4, pH 7.0. pH was varied from 4.5 to 9.0 using three buffers: pH 4.5 to 6.0 in 0.05 *M* sodium acetate; pH 6.0 to 7.5 in 0.05 *M* sodium phosphate; and pH 7.5 to 9.0 in 0.05 *M* Tris. Urea concentrations were varied from 0 to 4.8 *M* in 0.05 *M* sodium phosphate, pH 7.0.

Gel electrophoresis

Discontinuous gel electrophoresis was performed by a modification of the method of Laemmli (1970) either with or without sodium dodecyl sulfate. Acrylamide and N,N′-methylene-bisacrylamide concentrations for the separating gel were 5% and 0.13% (w/v), respectively, those for the stacking gel were 3% and 0.08% (w/v). Electrode buffer was 0.025 *M* Tris, 0.192 *M* glycine, pH 8.3. To estimate protein molecular weights, gels and reservoir buffer included 0.1% SDS. Prior to such electrophoresis, samples and molecular weight standards (Pharmacia Inc., Piscataway, New Jersey) were heated for three minutes at 100°C in 2% SDS and 5% β-mercaptoethanol. Insoluble material was removed by centrifugation at 12,000 × *g* for three minutes. Electrophoresis was carried out at 15 mA per slab for 30 minutes after which current was doubled for three hours. During electrophoresis the apparatus was maintained at ambient seawater temperature (approximately 15°C). Proteins were visualized by fixing and staining gels overnight at ambient temperature in 0.5% (w/v) Coomassie brilliant blue G-250 dissolved in methanol:acetic acid:water (40:15:45, v/v), and were subsequently destained first in methanol:acetic acid:water (45:10:45, v/v) and then in 5% acetic acid. Catechol oxidase activity was localized by immersing unfixed native gels for one hour in a solution of 1 m*M* 4-methylcatechol, 0.5 *M* NaCl, 0.05 *M* NaH_2PO_4, pH 7.0.

Results

The uterine flush, capsular wash and attachment fiber extract catalyzed the conversion of 3,4-dihydroxyphe-

nylalanine to dopaquinone. The oxidase in the uterine flush oxidized 4-methylcatechol, l-dopa and *N*-acetyldopamine. All preparations showed an initial low rate of oxidation which eventually increased and became linear at the later time points. Boiling the extracts for one minute destroyed this activity indicating the enzymic nature of the oxidizing principle. These measurements showed that an oxidase was associated with tanning capsules *in utero*. Since this enzyme could be flushed from the uterine lumen without mechanical disruption of the capsular material, the enzyme obtained must have been on the capsule surface, on the surface of the uterine epithelium, or free in the uterine lumen.

Extracts of nidamental glands and tanning capsules from spawning females oxidized a variety of catechols (Fig. 1). Ammonium sulfate fractionation of 1.0 *M* NaCl extracts resulted in a significant enrichment in enzyme specific activity (Table I for data on shell gland extract). Catechol oxidase activity was found predominantly in precipitates formed at 5 and 10% $(NH_4)_2SO_4$. Most of the protein precipitated at higher concentrations. Based on specific activity, the enzyme was purified 15–20 fold with respect to the initial homogenate. Since both the 5% and 10% ammonium sulfate precipitates contained active catechol oxidase, they were combined for further characterization of the enzyme.

Discontinuous gel electrophoresis (Fig. 2) showed that ammonium sulfate fractionation produced a substantial purification of the shell gland catechol oxidase. The precipitate formed in 5% ammonium sulfate consisted mostly of material aggregated at the bottom of the sample well and several proteins having an apparent molecular weight around 63,000 daltons (Lane 2, Fig. 2). Material precipitated by 10% ammonium sulfate also contained aggregates in the sample well (Lane 3, Fig. 2), in addition to a predominant band with an estimated molecular weight of 85,000 daltons. Using 4-methylcatechol as substrate, catechol oxidase activity in this 10% ammonium sulfate fraction was localized not to the major protein, but in a slower migrating diffuse band between 440,000 and 230,000 daltons. This band was not visible when stained with Coomassie blue (Fig. 2). This indicated that the active enzyme preparation contains relatively little protein having catechol oxidase activity and that the predominant protein could be a contaminant or a reduced or inactive form of the enzyme. Most of the protein in the original extract precipitated at ammonium sulfate concentrations higher than 10%, appearing predominantly in the 20% fraction (Lane 4, Fig. 2). These data confirm the substantial purification of the enzyme by ammonium sulfate fractionation.

Partially purified oxidases in 1.0 *M* NaCl extracts of tanning capsules and nidamental glands were compared with respect to substrate specificities. The two enzymes

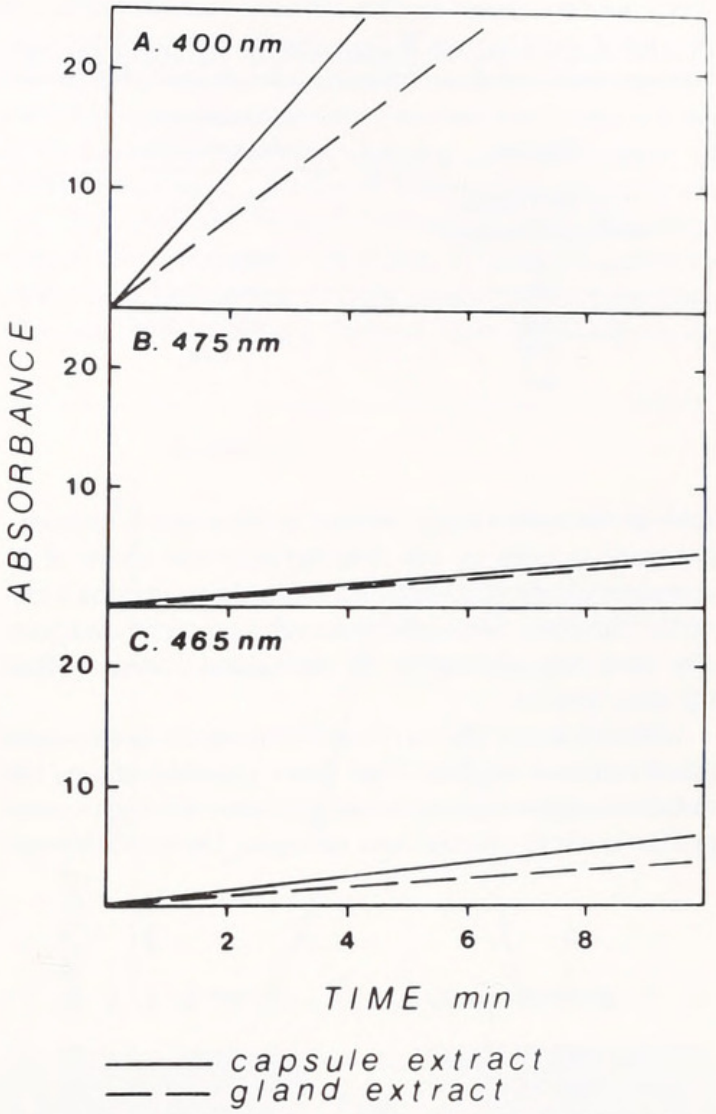

Figure 1. Oxidation of (A) 4-methylcatechol, (B) 3,4-dihydroxyphenylalanine, (C) 3-hydroxytyramine by partially purified extracts of tanning capsule and nidamental glands. Aliquots of redissolved 10% $(NH_4)_2SO_4$ precipitates of these extracts were incubated with 1 m*M* substrate in 0.5 *M* NaCl, 0.05 *M* NaH_2PO_4, pH 7.5 at ambient temperature. Absorbance was monitored at the indicated wavelengths. Values shown are means of triplicate analyses.

showed similar oxidative activities against 4-methylcatechol, 3-hydroxytyramine, and 3,4-dihydroxyphenylalanine (Fig. 1). Tyrosine was little affected by these enzymes.

The substrate specificity of the nidamental gland extract was examined in greater detail by determining its K_m and V_{max} for selected catecholic and phenolic compounds (Table II). Using the ratio V_{max}/K_m as an index of substrate preference (Segal, 1976), 4-methylcatechol was clearly the most favored substrate followed distantly by *N*-acetyldopamine and 3-hydroxytyramine. These data indicate that the nidamental gland enzyme has a strong preference for a methyl group substituted in *para* orientation to the first aromatic hydroxyl group. Chemical modification of the α-carbon by charged moieties

Table I

Catechol oxidase activity in ammonium sulfate fractions of the 1.0 M *NaCl extract of* Raja erinacea *shell glands*

Sample	Oxidase activity (μM/min/ml)	Protein (mg/ml)	Specific activity (μM/min/mg)	Purification factor (Fold)
1.0 *M* NaCl extract	26.4 ± 0.8	12.5	2.1 ± 0.1	1
$(NH_4)_2SO_4$ fractions:				
5%	152.4 ± 7.2	3.8	39.7 ± 1.9	18.91
10%	160.4 ± 5.1	5.1	31.4 ± 0.2	14.95
20%	40.4 ± 1.2	19.0	2.1 ± 0.1	—
30%	trace	9.0	—	—
40%	—	5.5	—	—

such as the carboxyls or amines of dopa and 3-hydroxytyramine or even by the *N*-acetylethyl side chain of *N*-acetyldopamine markedly diminished the enzyme's catalytic efficiency. Monophenols such as *p*-cresol and tyrosine were little affected by the shell gland enzyme within the assay period.

Table III shows effects of inhibitors on the nidamental gland catechol oxidase. Like other phenoloxidases, the nidamental gland enzyme was inhibited by oxygen competitors such as cyanide and nitrogen. Diethyldithiocarbamate, a metal chelator especially effective against copper-containing enzymes, also inhibited the enzyme.

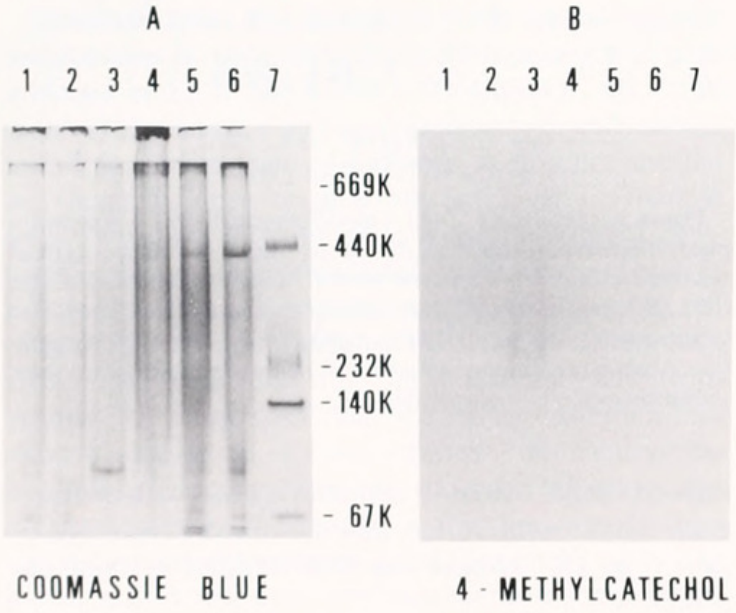

Figure 2. Polyacrylamide gel electrophoresis of ammonium sulfate fractions from nidamental gland extract. A. Coomassie blue R-250 stained SDS gel electrophoresis of mercaptoethanol reduced nidamental gland samples, as follows: Lane (1) original extract; (2) 5% ammonium sulfate precipitate; (3) 10% ammonium sulfate precipitate; (4) 20% ammonium sulfate precipitate; (5) 30% ammonium sulfate precipitate; (6) 40% ammonium sulfate precipitate; and (7) molecular weight standards as indicated (from highest to lowest: thyroglobulin, ferritin, catalase, lactate dehydrogenase, and bovine serum albumin). B. 4-methylcatechol (1 m*M*) staining of a gel without detergent was carried out for 1 h at pH 7.5 and room temperature. Sample lane order is the same as for Coomassie blue stained gel.

Oxidase activity was sensitive to the pH of the reaction mixture (Fig. 3). Both partially purified extracts from nidamental gland and tanning capsules exhibited maximal oxidation rates at pH 7.0–7.5. Little oxidation occurred at or below pH 5.0. Enzyme in the capsule extract was also sensitive to alkaline pH, retaining only a small portion of its activity towards 4-methylcatechol and l-dopa at pH 8.0–8.5 (Fig. 3). The nidamental gland enzyme was apparently less sensitive to alkaline pH. Oxidation rates of 4-methylcatechol by the partially purified nidamental gland enzyme were substantially above the natural oxidation rate of this substrate (Fig. 3). Even at pH 9.0 the enzyme retained some of its activity. At alkaline pH these catechols rapidly oxidize, so measurement of enzymic activity at pH 8.0 to pH 9.5 is only an estimate. While it is clear that oxidase activity from both capsule and nidamental gland declines above pH 7.5, some oxidative activity is retained at the pH of seawater (8.0–8.5).

The concentration of sodium chloride in the catechol oxidase assay was varied from 0.25 *M* to 1.0 *M* in 0.05 *M* NaH_2PO_4, pH 7.0. Enzymatic activity at NaCl concentrations below 0.25 *M* could not be accurately measured because of the substantial increase in turbidity re-

Table II

Substrate preference of shell gland catechol oxidase from Raja erinacea

Substrate	K_m (m*M*)	V_{max}*	V_{max}/K_m
p-Cresol	0	0	0
L-Tyrosine	0	0	0
Pyrocatechol	3.1 ± .05	6.9 ± .24	2.2
4-Methylcatechol	1.1 ± .19	45.5 ± .46	41.4
L-Dopa	1.37 ± .07	3.1 ± .20	2.3
Dopamine	0.11 ± .01	0.7 ± .10	6.1
N-Acetyldopamine	0.60 ± .22	4.3 ± .48	7.2

* μmoles oxidized/min/mg protein.
n = 3.

Table III

Inhibitors of shell gland catechol oxidase. 4-methylcatechol was used as substrate in all assays

Inhibitor	Type	K_i (μM)
Cyanide	Noncompetitive	4.02 ± .03
Diethyldithiocarbamate	Noncompetitive	166 ± .04
N_2	Probably n.c.	not measured

n = 3.

sulting from protein precipitation. NaCl concentrations above 0.25 *M* had little effect on the rate of oxidation of 4-methylcatechol by the nidamental gland extract (Fig. 4). A slight increase in the oxidation rate was observed at 0.5 *M* and this was statistically different from the rate at 0.4 *M* and 1.0 *M*.

Urea inhibited catechol oxidase in the partially purified nidamental gland extract in a concentration-dependent manner (Fig. 5). At the lowest concentration examined, 0.15 *M*, a slight reduction in oxidase activity was detected. Fifty percent inhibition occurred at approximately 4.0 *M* urea. At the concentration of urea generally maintained in elasmobranch tissues the oxidation rate of 4-methylcatechol was reduced by about 10%.

Typically oxidation did not commence immediately upon addition of the substrate, but rather occurred only after a brief delay. The duration of this delay was reduced by incubating the extract with α-chymotrypsin prior to adding substrate to initiate the reaction (Fig. 6). When crude nidamental gland extracts were stored at 4°C for several hours, their oxidative activity increased. These results suggest that the enzyme is produced in a latent form and that some endogenous factor in the crude extract is able to activate the latent enzyme.

Discussion

These observations confirm the presence of a catechol oxidase in tanning capsules and mature nidamental glands of the little skate, *Raja erinacea*, and thus support previous reports that this type of enzyme might be involved in the formation of elasmobranch egg capsules. We biochemically identified this enzyme as a catechol oxidase on the basis of its ability to catalyze the conversion of *ortho*-diphenols to their corresponding quinones. The nidamental gland enzyme is markedly inhibited by both cyanide and nitrogen, as expected of any oxidase. The enzyme is also inhibited by diethyldithiocarbamate which suggests that like other phenoloxidases it may contain copper. The enzyme prefers catechols bearing a methyl side chain which lacks exposed charged groups. These substrate prejudices are similar to those reported for catechol oxidases from mussel byssus (Waite, 1985) and periostracum (Waite and Wilbur, 1976). While the native substrate for the catechol oxidase in the egg capsule has not been characterized, we have recently detected 3,4-dihydroxyphenylalanine in hydrolyzates of freshly oviposited capsules of *Raja erinacea* (Cox *et al.*, 1987). In addition, Hunt (1985) has reported identification of three catechols, including 3,4-dihydroxyphenylalanine, in hydrolyzates of egg capsules of *Scyliorhinus canicula*. It is uncertain whether these catechols are in-

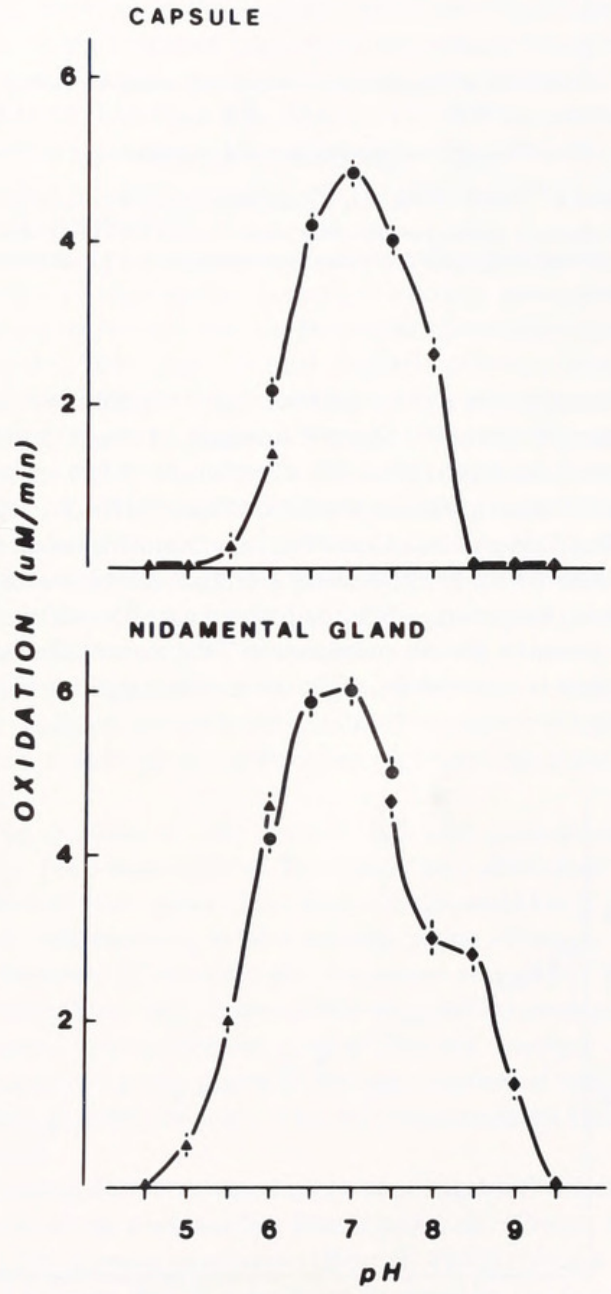

Figure 3. Effects of pH on oxidation rate of 4-methylcatechol by the capsule extract and nidamental gland extract. pH was varied from 5.0 to 8.5 using the three buffers: pH 4.5–6.0 in 0.05 *M* sodium acetate; pH 6–7.5 in 0.05 *M* sodium phosphate; pH 7.5–8.5 in 0.05 *M* Tris-HCl. Values shown are means of triplicate analyses of experimentals and boiled controls, and bars show the S.E.M.

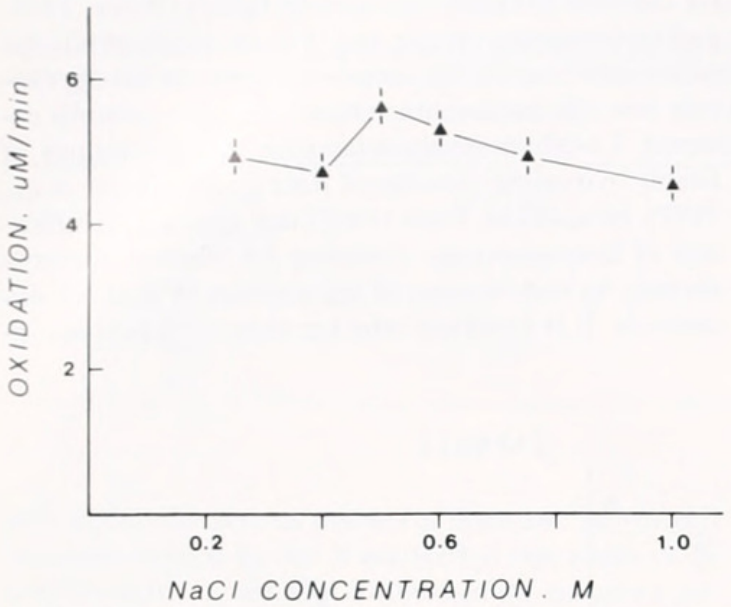

Figure 4. Effects of NaCl on the oxidation rate of 4-methylcatechol by the partially purified nidamental gland extract. NaCl was varied in 0.05 *M* NaH_2PO_4, pH 7.0. Values are means ± S.E.M. of triplicate analyses.

troduced as free amino acids or occur covalently bound to capsular proteins. Spectral analyses of intact capsular material suggested that the catechol in *Raja erinacea* capsules at oviposition is peptide bound (Koob, 1987). We have also shown that catechols are introduced into the capsular matrix following secretion and assembly of capsule precursors, while the formed capsules move into and reside in the uterine lumen. This accumulation of catechol is coincident with color development (Koob

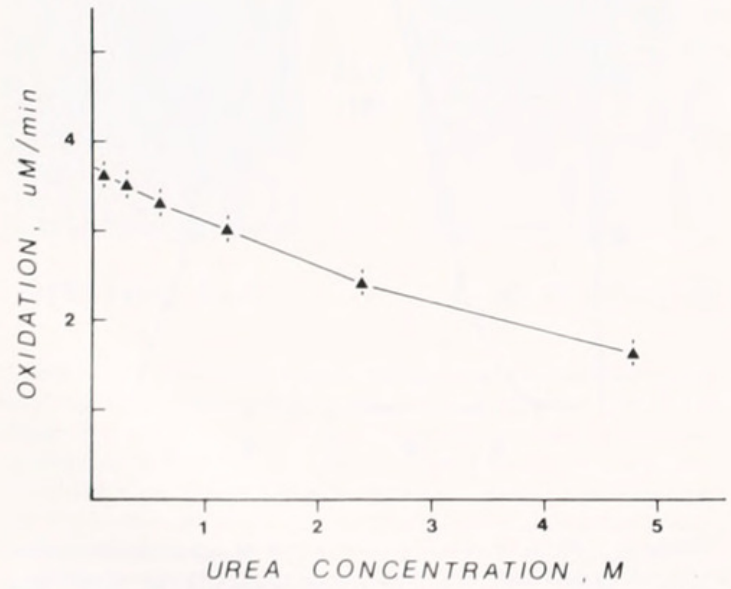

Figure 5. Effects of urea on the oxidation rate of 4-methylcatechol by the partially purified nidamental gland extract. Urea was varied in 0.5 *M* NaCl, 0.05 *M* NaH_2PO_4, pH 7.0. Values are means ± S.E.M. of triplicate analyses.

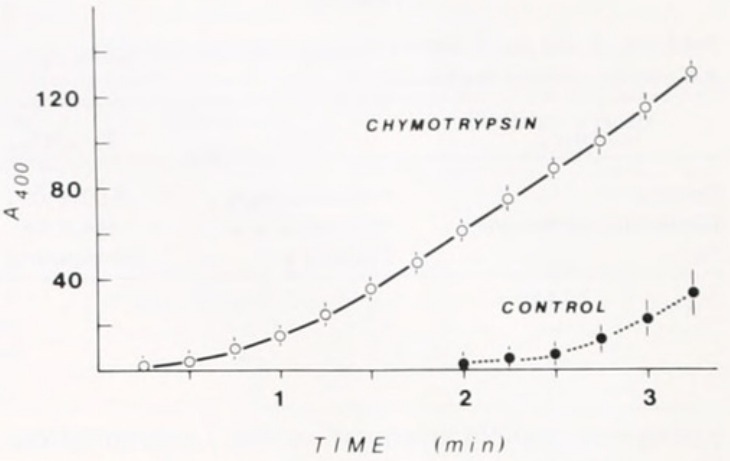

Figure 6. Initial rates of oxidation of 4-methylcatechol by extracts of nidamental glands with and without 40 μg of α-chymotrypsin. Aliquots of the 1.0 *M* NaCl extract were preincubated for 20 minutes at room temperature with 40 mg of a-chymotrypsin. Controls were incubated in parallel. Final assay conditions were 0.5 *M* NaCl, 0.05 *M* NaH_2PO_4, pH 7.5, and values presented are means ± S.E.M.

and Cox, 1986b). The presence of catechol oxidase in tanning capsules indicates that once catechols are introduced, they are susceptible to oxidation. These observations support Brown's (1955) original contention that this enzyme plays a pivotal role during the formation of skate egg capsules by catalyzing oxidation of catechols to highly reactive quinones forming dark pigments which tan the capsular matrix.

These experiments also establish the optimal conditions for assay of catechol oxidase from *Raja erinacea* nidamental glands. The partially purified enzyme exhibited maximal activity at 0.5 *M* NaCl and pH 7.0. Whether these conditions obtain in capsular material during tanning is not known, however, they closely resemble the osmolality and pH generally maintained in elasmobranch tissues. The sensitivity of the enzyme to urea is expected since renal and branchial enzymes from other elasmobranchs show identical inhibition by urea (Mályusz and Thiemann, 1976). We do not know whether urea is present in fluid bathing the tanning capsule or in the capsular material itself. The chemical conditions within the capsular matrix could be established during secretion of capsule precursors or alternatively could result from regulation of the intrauterine milieu.

The wide tolerance of nidamental gland catechol oxidase to alkaline pH and elevated salt concentrations provides evidence that the enzyme might remain active in seawater following oviposition of the capsule. Egg capsules of the little skate continue to tan during incubation by a process which may involve catechol oxidation (Koob, 1987). While it appears from the data presented here that catechol oxidase operates principally during capsular tanning *in utero,* it could also play a role in post-ovipositional tanning of the capsule.

One well characterized quinone tanning system that operates in seawater is the tanning of the attachment disc and byssus of *Mytilus edulis*. The byssal catechol oxidase displays optimum activity in salinity and pH near those of seawater (Waite, 1985). This enzyme's pH optimum (8.0) is slightly above that of the skate egg capsule enzyme, suggesting that the byssal enzyme may be more effective in seawater. Further study will be necessary to determine whether the egg capsule catechol oxidase in fact retains activity in seawater following oviposition. Such experiments will also furnish additional evidence regarding the role of this enzyme in the incubation-related tanning of the capsule.

The ability of α-chymotrypsin to shorten the delay in commencement of catechol oxidation by extracts of nidamental glands suggests that the egg capsule catechol oxidase is produced in a latent form which can be activated by proteolytic cleavage. Since this enzyme appears to auto-activate during storage as well as in the presence of substrate, these results also suggest that the gland produces a native activator. Whether the native activator resembles bovine α-chymotrypsin is not yet known. These observations are consistent with reports of latent phenoloxidases from both invertebrate and other vertebrate tanning systems.

For example, during sclerotization of the silkworm (*Bombyx mori*) cuticle, a latent phenoloxidase is activated by a serine protease (Dohke, 1973a, 1973b; Ashida and Dohke, 1980). A similar protease activates the prophenoloxidase of the arthropod immune response to invasive parasites (Ashida, 1971; Söderhäll, 1982; Ashida and Soderhall, 1984; Dularay and Lackie, 1985; Yoshida and Ashida, 1986; Saul and Sugumaran, 1987, 1988; for a review see Götz and Boman, 1985). In addition, *Mytilus edulis* produces a catechol oxidase that is latent towards catecholic substrates without prior activation by α-chymotrypsin (Waite, 1985). Among vertebrate tanning systems, the tyrosinase of amphibian skin is known to be produced in a latent form. Wittenberg and Triplett (1985a, b) have shown that detergents activate the latent tyrosinase from *Xenopus laevis*. This evidence is consistent with the preliminary data presented here. Together they implicate an activation process involving a zymogen of catechol oxidase during the formation of skate egg capsules.

Catechol oxidases have been detected in materials investing germ cells from many species and widely divergent taxa. Among fungi increases in phenoloxidase activity coincident with the development of fruiting-bodies have been noted for many species and have been investigated particularly in *Neurospora crassa* (Hirsch, 1954; Horowitz *et al.*, 1961), *Hypomyces solani* (Wilson, 1968), *Schizophyllum commune* (Phillips and Leonard, 1976, 1977; Leslie and Leonard, 1979), and *Agaricus bisporus* (Lindeberg, 1950; Turner, 1974; Rast *et al.*, 1981). Latent catechol oxidase has been identified as the predominant form of the enzyme in fruiting-bodies of *A. bisporus* (Yamaguchi *et al.*, 1970), though the mechanism of its activation has not been determined. The fruitbody phenoloxidase initiates melanization of propagule walls which is thought to confer protection from desiccation (reviewed by Sussman, 1968), lysis by other microorganisms (Potgieter and Alexander, 1966; Kuo and Alexander, 1967), and damage from ultraviolet light and other radiations (Sussman, 1968).

Seed coats of several wild legumes contain catechol oxidase. In *Pisum elatius*, for example, catechol oxidase activity in the seed coat rises sharply during the later developmental stages, especially during dehydration of the seed coat (Marbach and Mayer, 1975). The enzyme is believed to catalyze the generation of specific physicochemical properties important for dormancy and subsequent germination.

Catechol oxidases have also been detected in the eggshells and reproductive tracts of various invertebrates. Phenoloxidase activity has been histochemically demonstrated in both eggshells and vitellaria of many monogenetic and digenetic trematodes, and in certain cestodes (for a review see Clegg and Smyth, 1968; and Smyth and Halton, 1983). Recently, a dopa-rich eggshell precursor protein from the vitellaria of *Fasciola hepatica* has been purified and characterized (Waite and Rice-Ficht, 1987). Catechol oxidase activity has also been detected in the *Fasciola* vitellaria, and this enzyme has been partially purified (Cox and Waite, unpub. results). Like the elasmobranch egg capsule, the eggshells of many trematodes and cestodes appear to be stabilized by a form of sclerotization involving phenoloxidase catalyzed quinone tanning.

Egg cocoons of the leech *Erpobdella octoculata* undergo post-ovipositional hardening and darkening suggestive of some form of tanning. Knight and Hunt (1974) found the cocoons to be insoluble in the solutions used by Brown (1950a) with the exception of sodium hypochlorite. They also reported that the cocoons contain 3,4-dihydroxyphenylalanine and a catechol oxidase. Quinone tanning may occur in the egg cocoons of other annelids, but the evidence is as yet circumstantial (Brown, 1950b).

Among the Arthropoda, oothecae and left colleterial glands of the cockroaches *Blatta orientalis* (Pryor, 1940) and *Periplaneta americana* (Brunet, 1952) contain catechol oxidase which is believed to catalyze oxidation of 3,4-dihydroxybenzoic acid during the sclerotization of the ootheca. Likewise, eggshells of the house cricket, *Acheta domesticus*, reportedly contain a catechol oxidase (MacFarlane, 1960), while presence of the enzyme in shells of the mosquito, *Aedes aegypti*, has been in-

ferred (Walker and Menzer, 1969). Although several *o*- and *p*-diphenols have been extracted from eggshells and reproductive tracts of other insects, relatively little is known about the corresponding synthetic enzymes (for review of oothecal and eggshell proteins see Hinton, 1981).

To our knowledge, this is the first report of a catechol oxidase in egg capsules and oviduct of a vertebrate. The identification of catechol oxidase in egg capsules of *Raja erinacea* broadens the view that the products of this pathway possess particular properties adaptable to the needs of developing germ cells of fungi, plants, and animals.

Note added in proof

Inclusion of proteinase inhibitors (EDTA, benzamidine, N-ethylmaleimide and PMSF) during extraction of nidamental glands virtually eliminated catechol oxidase activity. The crude extract of one gland containing proteinase inhibitors contained less than 10% of the catechol oxidase activity found in the paired control gland extracted without proteinase inhibitors. These results support our suggestion that the egg capsule catechol oxidase is produced in a latent form requiring proteolytic activation.

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