

ELECTROCHEMICAL AND ENZYMATIC OXIDATION OF CATECHOLAMINES INVOLVED IN SCLEROTIZATION AND MELANIZATION OF INSECT CUTICLE*

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Abstract—The electrochemical properties of catecholamines that occur in the haemolymph and cuticle of *Manduca sexta* (L.) during development were studied. The cyclic voltammetric behaviour for dopamine, *N*-acetyldopamine (NADA) and *N*- β -alanyldopamine (NBAD) was similar except that the open chain *o*-quinones of the latter two compounds cyclized significantly more slowly than dopamine *o*-quinone. Tyrosinase from pharate pupal cuticle oxidized the three catecholamines with NBAD and NADA being the preferred substrates. Oxidation to the corresponding *o*-quinone, indolization and reoxidation to *p*-quinone imine was the pathway observed for both electrochemical and enzyme catalyzed reactions. The possible roles of these metabolites in cuticle sclerotization and melanization are discussed.

Key Word Index: Catecholamines, cyclic voltammetry, dopamine, *N*-acetyldopamine, *N*- β -alanyldopamine, tyrosinase, tobacco hornworm, *Manduca sexta*, sclerotization, melanization

INTRODUCTION

Several catecholamines are metabolized by insects for the sclerotization and pigmentation of cuticle (Brunet 1980). These include dopamine, *N*-acetyldopamine (NADA) and their glucoside and sulphate conjugates. Recently, *N*- β -alanyldopamine (NBAD) has been identified as the major catecholamine metabolite involved in pupal tanning of *Manduca sexta* (L.) and several other species of insects (Hopkins *et al.*, 1982). The specific metabolite utilized for tanning and the type of enzymatic oxidation of the substrate appears to determine the kind of cuticle formed and its pigmentation. Two primary pathways have been proposed for generating crosslinking agents in insect cuticle via the oxidation of catecholamines (Andersen 1979). One oxidizes the *o*-diphenol moiety (quinone sclerotization) and the other the aliphatic side chain (β -sclerotization). The catecholamine available may determine which pathway is utilized, i.e. NADA is a reactant for β -sclerotization, while β -alanine (or *N*- β -alanyldopamine) is associated with brown sclerotized cuticle crosslinked by quinone tanning (Andersen 1980, Brunet 1980). Insect melanins or black pigments may arise from the formation of indole

from DOPA or dopamine in the absence of acylation of the amino group. Quinone and indole quinone formation may be prevented by derivatization of an aromatic ring oxygen.

The present study was undertaken to compare the electroactivities of three of the major catecholamines involved in sclerotization or melanization of insect cuticle. Our goal is to determine the mechanism of oxidation of these catecholamines and to identify potential intermediates that may participate in cuticle morphogenesis. We have used cyclic voltammetry to observe the *o*-diphenol oxidative electron transfer and coupled reactions of dopamine, NADA and NBAD, and have also compared the *M. sexta* tyrosinase catalyzed reactions with the electrochemical oxidations.

MATERIALS AND METHODS

Chemicals

Dopamine and *N*-acetyldopamine were obtained from Sigma Chemical Co. or from Calbiochem. *N*- β -Alanyldopamine was prepared as follows: 1 g dopamine hydrochloride (Calbiochem) was dissolved in 50 ml of 10% (w/v) potassium tetraborate, pH 9.6. *N*- α -*t*-Butyloxycarbonyloxy- β -alanine-*N*-hydroxysuccinimide ester (1.43 g, Chemical Dynamics) was added slowly with vigorous stirring and left for 1 h at 25°C. During this time, the pH was maintained at 9.6 by the addition of 6 M KOH. The solution was then acidified to pH 1 with concentrated HCl and stirred for 30 min, during which time the boric acid gradually precipitated. After centrifugation at 9000 g and 4°C for 15 min the supernatant was applied to a column of Bio Gel P-2 (3.0 cm i.d. \times 170 cm) and eluted with 5% (v/v) acetic acid at a flow rate of 40 ml/h. Fractions containing *N*- β -alanyldopamine as determined by uv absorption were lyophilized and finally subjected to high performance liquid

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chromatography on a Bio Rad ODS-10 column (4 mm i.d. \times 250 mm) using a mobile phase of 0.2 M acetic acid-10% MeOH, pH 3 eluted at 25°C and a flow rate of 1 ml/min. The compound was >99% pure as determined by amino acid analysis after acid hydrolysis (Hopkins *et al.*, 1982).

Insects

Eggs of *Manduca sexta* (L.) were a gift from the Metabolism and Radiation Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota, U.S.A. Larvae were reared on a standard diet (Bell and Joachim 1976) and kept at 28°C and 60% relative humidity during a 16 h light-8 h dark photoperiod.

Tyrosinase preparation

Ten pharate pupal cuticles (6.8 g) were extracted with 60 ml 20 mM Tris pH 7.8 buffer containing 20% (w/v) sucrose, 1 mM diisopropylphosphorofluoridate and 0.1 mM phenylthiourea. The supernatant was collected by centrifugation at 9000 g and 4°C for 15 min and brought to 30% saturation with ammonium sulphate. The resulting precipitate was collected by centrifugation at 9000 g and 4°C for 15 min. The precipitate was dissolved in 12 ml of and dialyzed overnight against 10 mM Tris pH 8.5 containing 0.1 mM phenylthiourea. The dialysate was subjected to chromatography on Sephacryl S-300 (1.5 cm i.d. \times 82 cm) equilibrated with 0.1 M ammonium bicarbonate, pH 8.5. Tyrosinase fractions were assayed as described below, pooled, concentrated by ultrafiltration and subjected to rechromatography on Sephacryl. Tyrosinase activity was determined by a continuous colorimetric assay in 0.1 M sodium phosphate, pH 6.0 (Duckworth and Coleman 1970, Aso, Kramer, Hopkins and Whetzel, unpublished). Absorbance was monitored at 470 nm (dopamine) and 390 nm (NADA and NBAD). Velocity data were treated by Lineweaver-Burk and nonlinear least squares analyses.

Cyclic voltammetry

Cyclic voltammetric and chronoamperometric experiments were performed with a three-electrode potentiostat which incorporates circuits for electronic correction of ohmic potential loss between the reference and working electrodes (Bartak *et al.*, 1972). The potential of the carbon paste working electrode (area = 0.20 cm²) was relative to a saturated calomel reference electrode. A carbon rod served as the auxiliary electrode. The electrochemical studies were performed in an aqueous citrate-sodium phosphate buffer (McIlvaine 1921) pH 7.0. All solutions were deoxygenated with nitrogen prior to addition of the catecholamine.

RESULTS

Cyclic voltammetry of catecholamines

Cyclic voltammetry has been used to determine the electrode potentials at which catecholamines found in insect tissues undergo oxidation and to follow the fate of any new chemical species formed during the first potential scan. The cyclic voltammetric behaviour of dopamine at a carbon paste electrode is shown in Fig. 1A. On the first anodic scan, a peak (I) was observed at 0.21 V which corresponds to the two electron oxidation of dopamine (Fig. 2, 1, R=H) to the open-chain *o*-quinone (2, R=H). Upon reversal of the potential scan, the reduction of any unreacted dopamine quinone to dopamine was seen at 0.07 V (peak II). Relative to peak I, peak II was diminished in intensity, the difference being represented by another cathodic peak at -0.32 V (peak III). A third cathodic peak centered at -0.62 V was due to a background process. On subsequent cycles a second

anodic peak became prominent at -0.23 V (peak IV).

The results from cyclic voltammetry can be interpreted according to an oxidation-reduction pathway

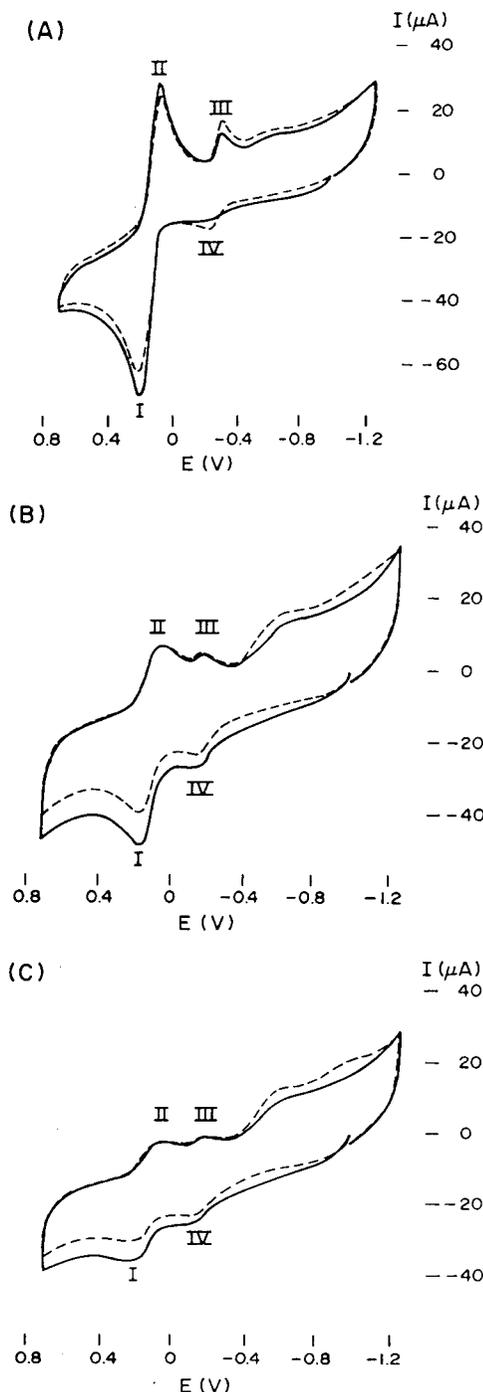


Fig. 1. Cyclic voltammograms of catecholamines at pH 7. Scan rate = 0.2 V/s. Initial cycle, —; second cycle, ----. The peak potentials less than -0.4 V were due to background processes. (A) Dopamine, 0.42 mM. (B) *N*- β -Alanyldopamine 0.25 mM after 15 min reaction with 1.6 μ g of the *Manduca sexta* cuticular tyrosinase preparation. (C) Same as (B) except after 1.5 h reaction. See text for experimental detail.

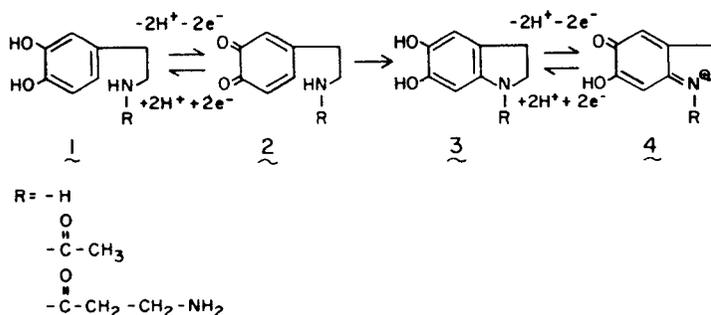
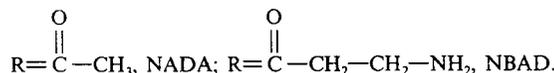
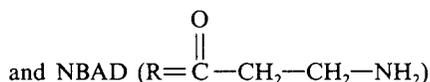
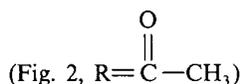


Fig. 2. Mechanism for oxidation of catecholamines and coupled intramolecular 1,4 addition reaction. 1, catecholamine; 2, *o*-quinone; 3, leucoaminochrome; 4, *p*-quinone imine. R=H, dopamine;



and an intramolecular 1,4-Michael addition reaction (Fig. 2, Bu'Lock and Harley-Mason 1951, Hawley *et al.*, 1967). The cyclization of compound 2 ultimately caused two new peaks to appear in the voltammogram. Peak III corresponds to the reduction of the cyclized oxidation product, *p*-quinone imine (4, R=H), to leucoaminochrome (3, R=H), while peak IV is the reoxidation of leucoaminochrome to *p*-quinone imine. Because the initial cyclized product (leucoaminochrome) is more easily oxidized than dopamine itself, the former can be oxidized by unreacted *o*-quinone (2). The oxidation of the catecholamine (1) to the *p*-quinone imine (4) is an overall four electron process and is an example of an electron transfer—chemical reaction—electron transfer (ECE) pathway.

The cyclic voltammetric behaviour of NADA



was also examined under the same reaction conditions; a summary of the cyclic voltammetric peak potentials for all three catecholamines and their corresponding quinones, leucoaminochromes and aminochromes (*p*-quinone imines) is given in Table 1. NADA and NBAD were oxidized at approximately the same potential as that for dopamine. The absence of a significant effect of *N*-acyl substitution upon the ease of catecholamine oxidation was expected since the electroactive *o*-diol function is effectively isolated electronically from the acylamine moiety. However, the *N*-acyl modification did have an important effect upon the rate of subsequent cyclization of the *o*-quinone. Although the cyclic voltammograms indi-

cated that all three catecholamines give basically the same electrochemical behaviour, the magnitudes of the anodic and cathodic peaks I and II for NADA and NBAD were nearly equal and the peaks for the corresponding aminochrome–leucoaminochrome redox couple (III and IV) were just discernible at a scan rate of 0.05 V/s. This behaviour demonstrated that the open-chain *o*-quinones of NADA and NBAD cyclize significantly more slowly than dopamine *o*-quinone. Since the first-order rate constant for the cyclization of the latter compound ($k = 38 \times 10^{-3} \text{ s}^{-1}$ at 25°C, Hawley *et al.*, 1967) represents the practical lower limit of rate constants which can be studied by electrochemical techniques at unshielded planar electrodes, only an upper limit for the cyclization rate constants for the remaining *o*-quinones can be given. On the basis of the relative magnitudes of peaks III and IV for the three catecholamines, the cyclization rate constants for the *o*-quinones of NADA and NBAD were estimated to be $< 10^{-3} \text{ s}^{-1}$. Thus, the electron withdrawing properties of the acyl substituents on the nitrogen of dopamine and their bulkiness decreased the rate of cyclization of NADA and NBAD by more than a factor of 40.

Previously, spectroscopic data indicated that NADA quinone does not undergo cyclization on the benzene ring because *N*-acylation renders the nitrogen non-nucleophilic (Graham and Jeffs 1977). However, the electrochemical behaviour of NADA reported here demonstrates that cyclization does indeed slowly occur.

Enzymatic oxidation of catecholamines

We next compared the enzymatic oxidation of the catecholamines with the electrochemical oxidation process. Tyrosinase from pharate pupal cuticle of the tobacco hornworm, *Manduca sexta* (L.), was incubated with NBAD at pH 7. The cyclic voltammogram

Table 1. Cyclic voltammetric peak potentials for catecholamines and their oxidation products*

Compound	Oxidation, $E_{p,a}$ (V)		Reduction, $E_{p,c}$ (V)	
	Catecholamine	Leucoaminochrome	<i>o</i> -Quinone	<i>p</i> -Quinone imine
Dopamine	0.21	-0.23	0.07	-0.32
<i>N</i> -Acetyldopamine	0.24	-0.24	0.02	-0.27
<i>N</i> - β -Alanyldopamine	0.18	-0.14	0.05	-0.18

*0.1 M citrate-phosphate buffer, pH 7.0 (McIlvaine 1921).

Table 2. Kinetic parameters for oxidation of catecholamines by *Manduca sexta* tyrosinase from pharate pupal cuticle

Substrate	K_m (mM)	V_{max} ($\mu\text{M}\cdot\text{min}^{-1}$)	V_{max}/K_m (min^{-1})
Dopamine	0.74 ± 0.11 (0.91)*	82.7 ± 3.7 (0.19)	0.11 (0.13)
<i>N</i> -Acetyldopamine	0.81 ± 0.10 (1.0)	436.0 ± 37.4 (1.0)	0.54 (0.62)
<i>N</i> - β -Alanyldopamine	0.41 ± 0.08 (0.51)	356.1 ± 39.4 (0.82)	0.87 (1.0)

* \pm SEM, $n = 5$. Numbers in parentheses compare substrate parameters with largest value set equal to 1.0.

for this enzyme-substrate mixture after 15 min reaction is shown in Fig. 1B. The solution became light brown during this time while the enzyme generated the quinone and quinone imine derivatives. The relative amounts of catecholamine:quinone:leucoaminochrome:quinone imine, as determined by peak intensities from the initial scan, were approximately 4:3:1:1. If the enzymatic oxidation did not afford the same intermediates as the electrochemical reaction, there would be no anodic peak (peak IV) in the first positive-going scan for the oxidation of the leucoaminochrome. A scan of the enzyme catalyzed reaction mixture was also taken after 1.5 h when the solution had become brown-red in color (Fig. 1C). The electroactive components were reduced in concentration and have apparently been converted to unknown electroinactive products. Because there were no new electroactive components in the enzymatic cyclic voltammogram, these results demonstrated that the initial products of the tyrosinase catalyzed reaction of NBAD are the same compounds generated by cyclic voltammetry. However the structure(s) of the compound(s) formed in the subsequent reactions of the aminochrome-leucoaminochrome is unknown. When the oxidation of the catecholamines is catalyzed by tyrosinase *in vitro*, both an enzymatic reaction and a chemical reaction take place. This type of mechanism may be denoted as enzymatic-chemical-enzymatic (EzCEz) by analogy with the electrochemical mechanisms literature. Whether a similar mechanism operates *in vivo* is unknown.

We also determined the relative rates of catecholamine oxidation by cuticular tyrosinase (Table 2). While the enzyme was quite effective at oxidizing all three catecholamines, the preferred order of enzymatic oxidation was NBAD > NADA > dopamine. No relationship between the rates of enzymatic oxidation and ease of electrochemical oxidation (NBAD > dopamine > NADA, Table 1) of the catecholamines was obvious. The lowest Michaelis constant was that for NBAD while NADA showed the highest maximum velocity. The K_m for NADA reacting with *M. sexta* tyrosinase (0.81 mM) was somewhat similar to that (0.53 mM) obtained with a phenoloxidase preparation from cuticle of *Calliphora vicina* (Barrett and Andersen 1981) but was smaller than that (1.3 mM) determined using cuticle phenoloxidase from *Schistocerca gregaria* (Andersen 1978).

While the three catecholamines associated with sclerotization and melanization of *M. sexta* were oxidized to *o*-quinones at similar rates electrochemically, the *N*-acylated compounds cyclized at significantly lower rates. These modified catecholamines however formed aminochromes faster

than dopamine in the presence of a cuticular tyrosinase. Apparently the enzyme facilitated both cyclization and oxidation of the substrates.

DISCUSSION

The significance of our results obtained from experiments *in vitro* with regard to the metabolism of the catecholamines *in vivo* remains to be determined since the cuticle incorporation mechanism of the electrophilic oxidation products into sclerotin and melanin is still uncertain. Because NBAD, NADA and dopamine are the major catecholamines at the time of cuticle morphogenesis in *M. sexta* (Hopkins *et al.*, 1982 and unpublished data) and are also excellent substrates for tyrosinase from cuticle, they are very likely the principal precursors of cross-linking agents for sclerotin and of polymerizing agents for melanin. Cuticular proteins, amino acids and other nucleophiles will presumably compete with the intramolecular cyclization pathway and form intermolecular homo- and hetero-adducts with the *o*-quinones. They may also condense with the cyclized ρ -quinone imines. The three *o*-quinones and the three ρ -quinone imines may all participate in cross-linking and polymerizing reactions. Their specific roles will be the subject of future investigations.

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