1992

A Comparison of Catecholamine Metabolism and Related Enzyme Activity in Parasitized and Nonparasitized Drosophila Melanogaster

Emily Vass
Loyola University Chicago

A COMPARISON OF CATECHOLAMINE METABOLISM AND RELATED ENZYME ACTIVITY IN PARASITIZED AND NONPARASITIZED *DROSOPHILA MELANOGASTER*

by

EMILY VASS

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF LOYOLA UNIVERSITY OF CHICAGO IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGY

MAY

1992

ACKNOWLEDGEMENTS

This study was successfully completed under the direction of Dr. Anthony J. Nappi, to whom I will always be thankful. I also wish to thank the other members of my committee, Drs. Albert Rotermund and Frederick H. Wezeman for their helpful comments and suggestions. I wish to thank Drs. Theodore Wright and Ellen Pentz, Department of Biological Sciences, University of Virginia for the *Ddc*^{ts2} and *Tyr-1* strains of *Drosophila melanogaster*. I am grateful to Dr. Manickam Sugumaran, Department of Biology, University of Massachusetts for providing some of the various standards that were screened. I also wish to thank Dr. Jianyong Li, Mrs. Francoise Frey, and Dr. Ann Faulkner for their assistance. I wish to dedicate this work to my best friend, JLS, whose patience, understanding, kindness, and encouragement were never ending.

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DA	Dopamine
DDC	Dopa decarboxylase
DHI	5,6-Dihydroxyindole
DOPAC	3,4-Dihydroxyphenylacetic acid
HPAC	p-Hydroxyphenylacetic acid
HPLC-ED	High pressure liquid chromatography with electrochemical detection.
HPPA	p-Hydroxyphenylpyruvic acid
MOPS	3[N-Morpholino]propanesulfonic acid
MPO	Monophenoloxidase
NAA	N-Acetylarterenone
NADA	N-Acetyldopamine
NADA-Q	N-Acetyldopamine quinone
NADA-QM	N-Acetyldopamine quinone methide
NANE	N-Acetylnorepinephrine
NAT	N-Acetyltransferase
NBAD	N-β-Alanyldopamine

NE	Norepinephrine
PTU	1-Phenyl-2-thiourea
TAT	Tyrosine aminotransferase

INTRODUCTION

Insects are capable of producing immune responses against a diversity of nonself components by invoking various humoral (Dunn, 1986; Boman and Hultmark, 1987; Hoffman and Hoffman, 1990) and cellular defenses (Salt, 1970; Rizki and Rizki, 1984; Gotz and Boman, 1985; Nappi and Carton, 1986; Christensen and Nappi, 1988; Sugumaran, 1990). Humoral responses are mediated by immune-like binding proteins (Soderhall et at., 1988; Johansson and Soderhall, 1989b; Reichhart, et al., 1989; Duvic and Soderhall, 1990) and inducible antibacterial peptides (diptericens, defensins, cecropins, hemolin, and eicosanoids) (Reichhart, et al., 1989; Duvic and Soderhall, 1990; Samakovlis et al., 1990; Sun et al. 1990; Ladendorff and Kanost, 1991; Stanley-Samuelson et al. 1991). Cellular responses employed by insects include phagocytosis and encapsulation (see reviews by Salt, 1970; Gotz and Boman, 1985). Foreign objects too large to be phagocytosed typically provoke a massive cellular encapsulation response which includes the aggregation and adhesion of numerous hemocytes (blood cells) around the foreign entity. A common feature in this type of cell-mediated response is the production of a brown to black pigment that occurs

in the innermost layer(s) of hemocytes. This pigment is generally considered to be melanin (or more specifically eumelanin) (Sealy et al., 1980), or a composite of melanin and sclerotin, although the physical and chemical properties of these capsules have never been adequately ascertained.

When larvae of *Drosophila* are invaded by parasitic wasps, their immune systems are challenged. In immune reactive hosts, the parasite becomes surrounded by a cellular pigmented capsule, and is destroyed by as yet unidentified cytotoxic components believed to be generated during the melanization process (Nappi, 1977; Poinar, 1979). The dead parasite sequestered in such a melanized hemocytic capsule, is retained inside the body cavity of the host throughout its development (Fig. 1). If however, the host fails to elicit an immune response, then the parasite develops successfully, emerging from the puparium of its host as an adult wasp (Fig. 1).

In *Drosophila*, larval defense reactions against foreign objects involve certain types of hemocytes or blood cells (Nappi and Streams, 1969). The types of hemocytes involved in the formation of multicellular capsules in *Drosophila* are lamellocytes and podocytes (Rizki and Rizki, 1984; Carton

and Bouletreau, 1985; Nappi and Carton, 1986). A third type of cell, the crystal cell, which is believed to contain tyrosine, may play an important role in the formation of melanin within the inner layers of the capsule. Recently, Nappi, et al., (1992) have demonstrated that the pigment formed is eumelanin, a heteropolymer comprised of various o-hydroquinones and o-quinones derived from tyrosine.

Certain catecholamines and their related metabolizing enzymes are implicated in invertebrate immune responses involving the sequestration of foreign invading organisms within cellular melanotic capsules. Some of these biogenic amines also are involved in cuticular sclerotization and wound repair processes (Lai-Fook, 1966). Since the biosynthesis of melanin is initiated by the enzyme phenol oxidase (tyrosinase (EC 1.14.18.1), considerable attention has focused on this enzyme system as a major component in insect cellular immune reactions (Sugumaran 1990). Recent studies have correlated enhanced phenol oxidase activity with the development of hemocytic pigmented capsules in response to eggs of insect endoparasites (Nappi et al., 1987, 1991; Li et al., 1989, 1991). This enzyme system can induce both the o-hydroxylation of monophenols (i.e. L-tyrosine and L-tyramine) to o-diphenols and the oxidation of diphenols (i.e.

L-dopa, dopamine (DA), N-acetyldopamine (NADA), N-β-alanyldopamine (NBAD)) to quinone. The quinone may then be non-enzymatically converted to melanin (Lerch, 1988) or enzymatically metabolized to melanin and/or sclerotin (Brown and Nestler, 1985; Andersen, 1985; Sugumaran, 1988). To date only a few studies have differentiated between mono- and diphenol oxidases (Nappi et al., 1987, 1991; Li et al. 1992).

Most of our knowledge about the phenol oxidase system in insects has developed from studies of cuticular sclerotization (Andersen, 1985) and from studies of enzyme deficient mutant strains of *Drosophila melanogaster* (Wright et al., 1976a, b, 1981, 1982a, b, 1987; Livingstone and Tempel, 1983; Rizki et al., 1985; Pentz and Wright, 1986; Pentz et al., 1990). Cuticle sclerotization and melanization involve the catabolism of tyrosine. Although sclerotization and melanization reactions are separate processes, occurring both simultaneously and independently in the same cuticle, they exhibit some similar biochemical transformations. The tyrosine derivatives that play a major role in cuticular sclerotization include NADA and NBAD and norepinephrine (NE). These sclerotizing precursors are oxidized by cuticular phenol oxidase(s) (principally tyrosinase (EC 1.14.18.1) or laccase (EC 1.10.3.2) to their corresponding reactive quinonoid compounds forming

adducts and crosslinks with protein-chitin matrices of the cuticle (Andersen, 1979, 1985; Brunet, 1980; Hopkins et al., 1982, 1984; Sugumaran, 1988. 1991). The sequence of reactions involved in melanogenesis includes an initial tyrosinase-catalyzed hydroxylation of tyrosine to dopa. Subsequently, dopa may be oxidized to form melanin, or decarboxylated to form DA. In one reaction pathway leading to melanin, dopa is oxidized to dopaquinone, which then undergoes nonenzymatic conversions to leucodopachrome and dopachrome. Subsequently, dopachrome is transformed into 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid by the action of dopachrome tautomerase (EC 5.3.2.3). This is followed by a tyrosinase-catalyzed oxidation of DHI to indole quinone, and polymerization of indolequinones to melanin via melanochrome (Fig. 2) (Hearnin and Tsukamoto, 1991). Dopamine may be either oxidized to dopaminequinone and then to indole-5,6-quinone to form melanin (via a reaction sequence similar to that followed by dopaquinone), or acetylated and then oxidized to form N-acetyldopamine quinone (NADA-Q) and ultimately sclerotin (Fig. 3). Some of the major enzymes involved in those processes include both mono- and di- phenol oxidases, decarboxylase, and N-acetyl-transferase.

Since melanization is a common feature in capsule formation, one

would expect to find alterations in both the levels of catecholamines and in the activities of the various enzymes involved in catecholamine metabolism during parasite encapsulation. However, studies concerning the changes in catecholamine profiles in infected insects have not been made, and research concerning the enzymes involved has only focused on the activities of phenol oxidase. Investigations of the involvement of other catecholamine-metabolizing enzymes have not been performed.

In *Drosophila*, as in other insects, certain catecholamines and other biogenic amines derived from tyrosine are developmentally and physiologically essential for various functions including neurotransmission (Livingstone, 1981), melanization and sclerotization of cuticle, female fertility (Wright, Steward et al. 1981), and immune responses involving the production of melanotic capsules around parasites (Nappi et al. 1991). Thus, when a melanotic encapsulation response is made, developmental processes that are dependent on common biogenic amines may be interrupted or alternated, at least transiently, for the synthesis of metabolites required for the more urgent immune response. As an approach to investigate this, the activities of four enzymes essential to catecholamine metabolism were studied in immune reactive larvae; tyrosinase (monophenol monooxygenase,

EC 1.14.18.1), dopa decarboxylase (DDC) (EC 4.1.1.26), N-acetyltransferase (NAT) (EC 2.3.1.5), and tyrosine aminotransferase (TAT) (EC 2.6.1.5). The copper-containing tyrosinase catalyzes three different reactions in the biosynthetic pathway to melanin: 1) the initial hydroxylation of tyrosine to dopa; 2) the oxidation of dopa to dopaquinone; and 3) the oxidation of DHI to indole-quinone. The enzyme DDC catalyzes the decarboxylation of dopa to DA, an important precursor not only for the synthesis, by NAT, of N-acetylated catecholamines that form sclerotin, but also for melanin (Kramer et al.1984; Hiruma et al. 1985; Roseland et al. 1987). The activity of TAT is potentially important to an immune response, since during early larval development tyrosine is metabolized mainly by deamination (Sekeris and Fragoulis, 1989), and perhaps also by decarboxylation, and the products of these reactions (p-hydroxyphenylpyruvic acid (HPPA) and tyramine, respectively) converted to p-hydroxyphenylacetic acid (HPAC) (Fig. 4). Toward the end of larval development the metabolic pathway involving the initial hydroxylation of tyrosine and leading to cuticular melanization and sclerotization becomes more active in preparation for the formation of the puparium. Under conditions that favor hydroxylation three important o-diphenols may be

produced; dopa from tyrosine, DA from tyramine, and 3,4-dihydroxyphenylacetic acid (DOPAC) from either dopa, DA, or HPAC (Fig. 4).

The purpose of this study was to identify the free, unconjugated hemolymph catecholamines formed in immune reactive *Drosophila* larvae, and to elucidate the roles of various catecholamine-metabolizing enzymes in the cellular defense response of this insect. As an approach, strains of *D. melanogaster* that exhibited opposite immune capabilities against the endoparasitic wasp *Leptopilina boulardi* were used. The host strains were selected from various isofemale lines, each derived from a single female inseminated in the wild (Brazzaville, Africa) for their ability (reactive strain, R) or inability (susceptible strain, S) to produce an immune reaction against a sympatric strain of *L. boulardi.* To evaluate the participation of the enzymes DDC and phenol oxidase in melanotic encapsulation, two genetically-defined enzyme deficient mutants of *D. melanogaster* were employed, Ddc^{ts2} and *Tyr-1*. The DDC deficient *Drosophila* strain used is a temperature sensitive mutant (Marsh and Wright, 1980). At the permissive temperature of 20°C, the mutant has normal enzyme activity. However, at the restrictive temperature of 29°C, DDC activity is reduced by

90%. The *Tyr-1* mutant possessed a deficiency in phenol oxidase, exhibiting only 10% of normal enzyme activity (Pentz et al., 1990). Studies utilizing this mutant evaluated whether reduced phenol oxidase activity was sufficient to elicit an immune response. The encapsulation responses of each mutant were evaluated after parasitization by the wasp *Leptopilina boulardi*.

For comparative purposes, we also investigated whether the nonreactivity of the S strain against *Leptopilina* was due to a dysfunctional immune system, unable to recognize nonself components, or if host susceptibility was in fact specific for this one species of parasite. To examine the basis for this susceptibility in the S strain, we used a second species of parasitic wasp, *Asobara tabida*.

Insects. The *D. melanogaster* used in this study were raised on standard cornmeal and yeast medium. A Brazzaville strain of *D. melanogaster* was used to rear and to maintain at 25°C a sympatric strain of the parasitic wasp *L. boulardi*. A wild strain of *D. melanogaster* was used to rear a second species of wasp, *A. tabida*. Adult wasps were fed a 50% honey solution and kept at 18°C. Four host strains with differing immune capabilities against *L. boulardi* were used to study catecholamine changes during infection; a highly immune reactive strain (R-445), a virtually totally susceptible or (nonreactive) strain (S-22) (Carton and Bouletreau, 1985; Carton and Nappi, 1991; Nappi et al., 1991), and two enzyme deficient strains, Ddc^{ts2} and *Tyr-1*. The Ddc^{ts2} strain is temperature sensitive, and at the restrictive temperature of 29°C it exhibits less than 3% of the normal DDC activity (Wright et al, 1981; Tempel et al., 1984). The *Tyr-1* strain is a phenol oxidase deficient strain that possesses less than 10% of the normal enzyme activity (Pentz et al., 1990).

Chemicals. 5, 6-dihydroxyindole (DHI) was obtained from Regis Chemical, Morton Grove, Illinois. Arterenone, N-acetylarterenone (NAA),

N-acetylnorepinephrine (NANE), NBAD, and N-β-alanylnorepinephrine were gifts from Dr. M. Sugumaran of the Department of Biology at the University of Massachusetts at Boston, Massachusetts. All other reagents used were obtained from Sigma Chemical Company (St. Louis, MO).

Infection of Host Larvae. All host strains were parasitized by exposing fly larvae to several female wasps during a 4 hr period. Except for the temperature sensitive DDC mutant, Ddc^{ts2} , the hosts were reared at 25°C, and the age of the host at the time of parasitization was 50 $\pm$ 3 hr. Ddc^{ts2} larvae reared at the permissive temperature of 20°C were parasitized at a host age of 72 $\pm$ 3 hr, while Ddc^{ts2} larvae reared at the restrictive temperature of 29°C were parasitized at 50 $\pm$ 3 hr. Under these two different temperature conditions the Ddc^{ts2} larvae were at a comparable stage of development when parasitized. Control experiments were conducted with wild-type larvae maintained at the same temperatures as mutant strains.

Hemolymph Catecholamines. The term hemolymph used in this study refers to both the cellular (hemocytes) and noncellular (plasma) components of the blood. Hemolymph samples were taken from nonparasitized (control) and parasitized larvae at specified times post-infection. Larvae were first removed from the culture medium, washed in MOPS buffer, pH 6.5, and

then dried on filter paper. Hemolymph was extracted from individual larvae by making a small incision in the cuticle just in front of the caudal spiracles. Hemolymph issuing from the wound was collected with a microcapillary pipette and diluted (1:50 v/v) with 0.8 M citric acid stop buffer (pH 2.4) for analyses of hemolymph catecholamines. Immediately after individual hemolymph samples were taken they were frozen at -20°C, and each host larva dissected and examined to verify the presence of the parasite and the host immune response, if manifested. Under these conditions catecholamines were stable for several months. When thawed, a 5 μl aliquot of each sample was examined and a catecholamine profile obtained by high pressure liquid chromatography with electrochemical detection (HPLC-ED). Subsequently, blood samples from immune reactive larvae were pooled to obtain sufficient volumes for multiple analyses that were required to identify endogenous electroactive components. Pooled hemolymph samples were also obtained from non-infected controls and from susceptible hosts that exhibited no immune response.

HPLC-ED Analyses. Hemolymph samples for catecholamine determinations were analyzed electrochemically following separation by reverse phase, ion-pairing HPLC-ED. The HPLC-ED system consisted of

a Bioanalytical Systems (West Lafayette, IN) LC-4B electrochemical detector equipped with a glassy carbon electrode maintained at potentials of +750 and +850 mV versus a Ag/AgCl reference electrode and a sensitivity range of 2 to 20 nA. Separations were achieved at 40°C by a BAS Phase-II, 3-μm ODS reverse phase column (3.2 mm I.D. x 10 cm). A Gilson (Madison, WI) 712 HPLC System Controller was used to integrate peak dimensions. Two different chromatographic conditions were used to verify the identity of the electroactive components in the hemolymph that co-eluted with authentic standards. Among the 50 authentic standards used for this purpose were the principal deaminated, transaminated, and O-methylated derivatives of tyrosine, tyramine, DA, dopa, and NE (Table 2). The first mobile solvent system was comprised of 0.1 M citrate buffer (pH 2.9) containing 5% acetonitrile, 0.5 mM sodium octylsulfate, and 0.7 mM Na_2EDTA. The second mobile phase was comprised of 50 mM citric acid (pH 3.2), 2.5% acetonitrile, 1 mM sodium octylsulfate, and 0.7 mM Na_2EDTA. The flow rate was maintained at 0.8 ml/min. Attempts to identify unknown electroactive components in samples of hemolymph and in reaction mixtures were first made by comparing retention times of these components with those of authentic standards. Subsequently, the

components were identified if they co-eluted with the authentic standards under two different chromatographic conditions, and if their peak dimensions were amplified in proportion to the amount of standard added to the hemolymph sample.

Enzyme Preparations. Enzyme activity was studied using samples of larval hemolymph, and the supernatant of whole body extracts. Hemolymph was removed from a small incision made in the larval cuticle. The blood issuing from the wound was collected with a microcapillary pipette and diluted (1:50 v/v) with MOPS buffer and frozen at -70°C for subsequent analyses of enzyme activity. The supernatant of larval homogenates was prepared by removing 12 larvae at various stages of development and at specified intervals after parasitization, and washing them in 0.2 M phosphate buffer. The larvae were then transferred into 0.4 ml of ice-cold buffer (either 50 mM MOPS, pH 6.5 or 200 mM potassium phosphate buffer, pH 7.3), and frozen at -20°C. Immediately before each enzyme assay the frozen samples were thawed on ice and homogenized. Homogenization was performed for 3-5 min in ice-cold buffer that contained 0.1% Triton X-100 (plus 0.2 mM 1-phenyl-2-thiourea (PTU) to inhibit tyrosinase in assays of DDC, TAT, and NAT). The homogenate was

centrifuged at 4°C for 10 min at 15,000 g and the supernatant fraction recentrifuged under the same conditions. The resulting supernatant served as the enzyme preparation and was stable for at least 2 months when stored at -70°C.

Enzyme Assays.

1. General Procedures.

The reaction mixtures were incubated at 30°C for varying periods up to 60 minutes. At specified intervals enzyme activity was stopped by removing 40 μl aliquots and placing them into 160 μl ice-cold 0.8 M citrate stop solution (pH 2.4). The stopped reaction mixture was centrifuged for 15 min at 15000 g at 4°C and 5 μl samples of the supernatant were then analyzed by HPLC-ED for enzyme activity. Standard curves for the appropriate substrates and products were prepared for each assay. The sensitivity of the electrochemical detector was monitored prior to and at the end of each assay. Column retention times and linear detector responses were periodically calibrated using external standards in order to accurately determine product formation for enzyme activity. Reaction velocities were calculated by measuring changes in the peak dimensions (height and/or area) of the formed product, and calculating concentrations based on standard

curves and analyzed by linear regression. The latter were corrected for percent recovery of internal standards (3,4-dihydroxybenzylamine or α-methyldopa) which were incorporated into various stopped reaction mixtures and run prior to and following each assay. To accurately assess activity all enzyme preparations were analyzed separately prior to each assay to establish base-line, endogenous levels of the substrates and reaction products. Linearity for each enzyme was determined by incorporating into the standard reaction mixture increasing amounts of enzyme preparation. Specific enzyme activity is expressed as the rate of product formed per mg protein. Protein concentrations were determined using the Bradford (1976) microassay with bovine serum albumin used as the standard.

2. Monophenol Oxidase Activity.

Monophenol oxidase (MPO) activity was determined by a slight variation of a recently developed specific HPLC-ED method that was determined to be at least 100 times more sensitive than radiometric and spectrophotometric methods, and especially suitable for small sample volumes and low levels of enzyme activity (Li and Nappi, 1991). The reaction mixture, final volume 0.3 ml, was comprised of 0.45 μmol of substrate (tyrosine or tyramine), 0.16 μmol ascorbic acid, 1.2 pmol of the

cofactor NADA, and 50 μl of enzyme preparation. In all control mixtures 50 μl MOPS buffer substituted for the enzyme preparation.

3. N-acetyltransferase Activity

NAT activity was measured by a modification of the assay of Li and Nappi (1992). The reaction mixture contained, in a total volume of 0.3 ml, 50 mM MOPS buffer, pH 6.5, 0.23 μmol substrate (DA or NE), 0.23 μmol acetyl Coenzyme A, and 7.5 pmol of PTU to inhibit phenol oxidase. In control incubations buffer substituted for either enzyme or acetyl CoA, or the latter was replaced by acetyl coenzyme A.

4. Dopa Decarboxylase.

DDC catalyzes the conversion of dopa to DA. The activity of DDC in *Drosophila* was assessed during the third stage of larval development by electrochemical detection of the reaction product. Reaction mixtures were comprised of 50 mM MOPS buffer (pH 6.5), 40 μl enzyme preparation (0.1 μl hemolymph), 70 nmol L-dopa, 0.56 nmol pyridoxal phosphate, and 28 nmol PTU in a final volume of 140 μl. Incubations were performed at 30°C. In control incubations buffer substituted for the enzyme preparation.

5. Tyrosine Aminotransferase

TAT (tyrosine:2-oxoglutarate aminotransferase) catalyzes the

conversion of tyrosine to the α-keto acid HPPA. In *Drosophila* larvae this transformation to the α-keto acid was found to be accompanied by decarboxylation to hydroxyphenylacetic acid (HPAC). Thus, this coupled enzyme activity was measured by a modification of established but less sensitive spectrophotometric methods (Diamondstone 1966; Tauber and Hardland, 1977), and was based on electrochemical detection and quantification of HPAC. The reaction mixture contained, in a total volume of 0.3 ml, 0.2 M potassium phosphate (pH 7.3), 1.5 mM tyrosine, 5 mM α-ketoglutarate, 0.4 mM pyridoxal-5'-phosphate, 25 μM of PTU to inhibit phenol oxidase, and 50 μl enzyme extract prepared as described above.

Statistical Analysis. Results are presented as the means ± SEM of the determinations specified. Differences between mean values were evaluated using the Student t-test, and the Duncan's new-range multiple comparisons test. Difference between two means was considered significant when $P < 0.05$.

1. Immune Interactions of *D. melanogaster* Against *L. Boulardi.*

In immune reactive hosts the egg of *L. boulardi* was rapidly enveloped in a melanotic capsule that was seen readily within the body cavity of the host where it remained throughout the development of the fly (Fig. 5). In susceptible i.e., non-reactive hosts, the parasites developed and emerged as adults from the host puparium.

In this study, second stage larvae (50 hr) from five strains of *D. melanogaster* were exposed to 4-6 female *L. boulardi* for a period of 4 hr. Following parasitization, host larvae from each group were given ample time (approximately 48 hr post-infection) to develop an immune response, and then dissected when melanotic encapsulations were manifested. The percentages of encapsulation for each host strain of *Drosophila* against the *L. boulardi* are given in Table 1. Approximately 80% of the eggs of *L. boulardi* were encapsulated in each of three host strains, R strain, *Tyr-1*, and Ddc^{ts2}, but in the latter case only at the permissive temperature of 20°C. The lowest rate of encapsulation (approximately 3%), was recorded in the S-strain. At the restrictive temperature (29°C), less than 9% encapsulation

was seen in the *Ddc*^{ts2} mutant (Table 1).

2. Detection of Hemolymph Catecholamines

Approximately 20 hr after infection samples of hemolymph were taken from parasitized larvae of each host strain in order to identify catecholamines produced during immune reactions. Three replicate tests were performed with non-parasitized larvae as controls, and a total of approximately 700 samples of hemolymph were assayed by HPLC-ED. Representative chromatographic profiles of the catecholamines found in samples of hemolymph from both control and parasitized immune reactive larvae at 20 hr post-infection are presented in Figure 6. Depending upon the stage of infection, HPLC-ED analyses made at a potential of + 700 mV showed hemolymph from immune reactive individuals to be comprised of up to five electroactive components with retention times of 1.4, 1.8, 2.6, 3.2, and 3.6 min. At a potential of + 850 mV, an additional component appeared at 3 min.

In nonparasitized control larvae of both immune reactive and susceptible strains, two distinct electroactive components were detected with elution times of 1.8 and 2.8 min (Fig. 6A). A similar chromatographic profile was exhibited in hemolymph from parasitized larvae of the susceptible strain in

which hosts the parasite develops successfully with no evidence of a pigmented encapsulation response (Fig. 7).

3. Electrochemical Determination of Hemolymph Components.

Hemolymph samples from nonparasitized controls and from parasitized immune reactive and susceptible host strains, were pooled separately for identification of the hemolymph components by HPLC-ED. The elution times of the hemolymph catecholamines were compared under identical conditions with approximately 50 authentic standards including the principal deaminated, transaminated, and O-methylated derivatives of tyrosine, tyramine, DA, dopa, and NE (Table 2). Co-injection experiments with standards that co-eluted with unknown components were subsequently performed using two solvent systems. In these experiments, verification of the components was made when their peak dimensions increased in proportion to the amounts of authentic standard incorporated into the sample. In immune reactive larvae the hemolymph components identified by these methods were dopa (1.8 min), NAA (2.6 min), tyrosine (3 min) and 5, 6-dihydroxyindole (DHI) (3.2 min) (Fig. 8). The components eluting at 1.4 and 3.6 min were not identified. Hemolymph from non-parasitized larvae and from susceptible hosts where no immune reaction occurred did

not contain NAA or DHI, but did contain both dopa (1.8 min) and DOPAC (2.8 min). The latter component was not present in immune reactive hosts at the times examined.

The blood samples were pooled from larvae of the immune reactive strain at various intervals post-parasitization and were analyzed by HPLC-ED to ascertain relative changes in the amounts of hemolymph catecholamines at different times during the pigmented encapsulation response against *L. boulardi* (Fig. 9). Essentially the same chromatographic profiles were found in immune reactive larvae at each interval post-infection. In immune reactive larvae dopa levels were highest in the pooled samples at 24 h post-infection, during which time eggs of the parasites were being sequestered by pigmented hemocytic capsules that were clearly visible within the hemocoels of the host larvae. Also during this stage the level of DHI was highest in comparison to other post-infection assay periods. NAA and DHI were found only in parasitized larvae of the immune reactive strain, while DOPAC was found only in hemolymph from nonparasitized control larvae and in parasitized larvae of the nonreactive, susceptible strain.

4. Immune Competence of S-strain Against *Asobara tabida*.

Since in S strain hosts the parasitic wasp *L.boulardi* developed

unmolested with no evidence of a melanotic encapsulation response, it was questioned whether this host strain lacked an adequate recognition mechanism, or if host susceptibility was specific for this one species of parasite. To investigate these possibilities a second species of parasitic wasp, *Asobara tabida*, was employed, and comparative studies made by infecting both R and S strain hosts. Against *A. tabida* both R and S strains were found to be highly immune reactive exhibiting encapsulation rates of approximately 90 and 95%, respectively (Table 3).

Although the susceptibility of S strain larvae appeared to be specific to *Leptopilina*, we were interested in knowing if the immune response made by S strain hosts was in fact the same as that made by R strain hosts. Samples of hemolymph were collected at 6 hr intervals from host larvae 24-48 hr post-infection, during which time melanotic capsules were forming around the parasite eggs. When analyzed by HPLC-ED the S strain hosts that encapsulated *Asobara* were found to have chromatographic profiles identical to those of immune reactive R strain larvae (Fig. 10). Thus, R and S strain hosts use similar biochemical mechanisms in their defense responses against different parasites.

5. Activities of Catecholamine-Metabolizing Enzymes.

Comparative studies were made of the changes in the activities of four catecholamine-metabolizing enzymes during parasitization. Enzyme activity was monitored at 24 and 30 hrs post-infection, at a time when melanotic encapsulation responses against the eggs of the parasites were forming and could be readily detected. R strain and *Tyr-1* strain larvae which exhibited extensive melanotic encapsulation responses were employed for studies of MPO, NAT, and TAT activities, and the Ddc^{ts2} mutant strain was used for examining alterations in DDC activity. The suitability of each enzyme assay was established in a series of initial studies employing alternate substrates and various concentrations of cofactors and enzyme inhibitors.

Prior to conducting the enzyme assays, samples of hemolymph from each study interval were examined to determine endogenous levels of the substrates and/or products of reactions to be measured in the assays. Under the same electrochemical conditions that were used in subsequent enzyme studies no endogenous DA, NADA, or tyramine were detected. However, dopa and tyrosine were detected.

Monophenol Oxidase. The initial and rate-limiting reaction in the metabolism of catecholamines is the hydroxylation of tyrosine to dopa.

Because of the high levels of endogenous dopa present in the samples of hemolymph, MPO activity was monitored by examining the hydroxylation of the related monophenol, tyramine, which formed DA. Under the specified assay conditions, both the product (DA) and substrate were readily differentiated from each other and from the coenzyme NADA (Fig. 11). The retention time for DA was 5.3 min and for tyramine 8.1 min. The retention time for NADA was 3.6 min.

Enzyme activity was found to be linear in time (up to 30 min) varying proportionally with the concentrations of enzyme and substrate. In nonparasitized larvae there was little change in the rate of MPO activity during the study interval, averaging 0.98 pmol/min/mg protein (Fig. 12). In immune reactive larvae the mean rate of MPO activity in R strain and *Tyr-1* strain hosts was approximately 10 times higher than in the controls, averaging 10.3 and 9.55 pmol/min/mg protein, respectively at 24 hrs post-infection ($p < 0.01$). At 30 hrs post-infection MPO activity had returned to more normal levels, but was still significantly higher ($p < 0.05$) in both host strains (about 3.8 pmol/min/mg protein) than in the controls (Fig. 12).

N-Acetyltransferase. NAT activity could be assessed by measuring either the acetylation of either DA or NE. The elution times for both

substrates and their products are given in Figure 13. The assay chosen for this investigation employed NE as substrate and measured the production of NANE, since the presence of DHI (elution time 3.3 - 3.4 min) in immune reactive larvae interfered with the measurement of NADA (elution time 3.4 - 3.5 min) (Fig. 13), but not NANE (Fig. 14). Activity of the enzyme was found to be linear in time (up to 20 min) varying proportionally with the concentrations of enzyme and substrate ($p < 0.05$). The activity of NAT in immune reactive hosts was significantly lower than non-parasitized control larvae 24 hrs post-infection, but virtually identical to the control larvae 6 hrs later (Fig. 15). The mean rate of NAT activity in control larvae was 2.3 nmol/min/mg protein, and 2 to 5 times less in immune reactive larvae of R and *Tyr-1* strains, respectively, 24 hr post-infection.

Tyrosine Aminotransferase. TAT activity was readily detected by monitoring the production of HPAC (Fig. 16). Product identification was established by co-injection experiments (Fig. 17). However, the intermediate in the reaction pathway, HPPA, was not detected by the electrochemical methods employed.

As was seen with NAT, a similar but less pronounced decrease in enzyme activity was seen with TAT 24 post-infection. At this time immune

reactive individuals of both R and *Tyr-1* strains exhibited approximately 2/3 the TAT activity that was found in controls (Fig 18). At 30 hrs post-infection reaction rates of TAT activity in parasitized larvae were not significantly different than the controls.

Dopa Decarboxylase. The activity of DDC was assayed in the Ddc^{ts2} mutant larvae to determine whether the immune incompetence exhibited by these larvae when reared at the restrictive temperature of 29°C (Table 1) was in fact associated with a lower level of enzyme activity. Hemolymph samples were taken at a time that corresponded to the early development of melanotic capsules in Ddc^{ts2} hosts parasitized by *L. boulardi.* Enzyme assays were run at 5, 15, 30 and 45 min incubations, and the rate of activity was found to be linear over this time range. Under restrictive conditions, the mean specific activity of DDC in Ddc^{ts2} mutants was 0.74 nmol/min/mg protein, compared to controls which averaged 1.7 nmol/min/mg protein ($p < 0.05$).

Figure 1. Developmental fates of the parasitic wasp *Leptopilina boulardi* in two strains of *Drosophila melanogaster*. In the immune reactive (R) strain the egg of the parasite is sequestered in a melanotic capsule that is retained within the body cavity throughout development. In the susceptible (S) strain the parasite develops and emerges as a adult.

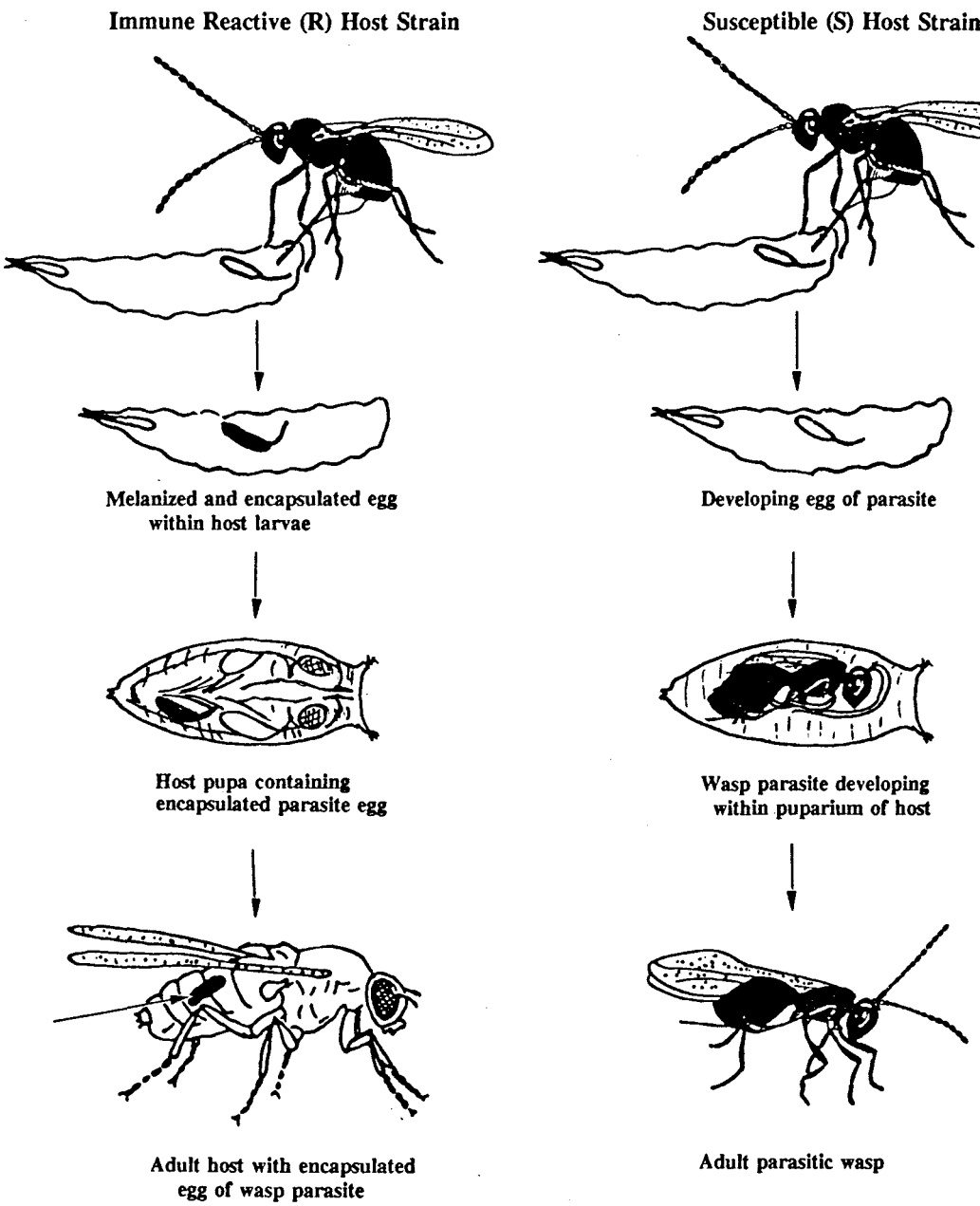
Immune Reactive (R) Host Strain
Susceptible (S) Host Strain
Melanized and encapsulated egg within host larvae
Developing egg of parasite
Host pupa containing encapsulated parasite egg
Wasp parasite developing within puparium of host
Adult host with encapsulated egg of wasp parasite
Adult parasitic wasp

Figure 1.

Figure 2. Major pathways involved in the biosynthesis of eumelanin, phaeomelanin, and sclerotin.

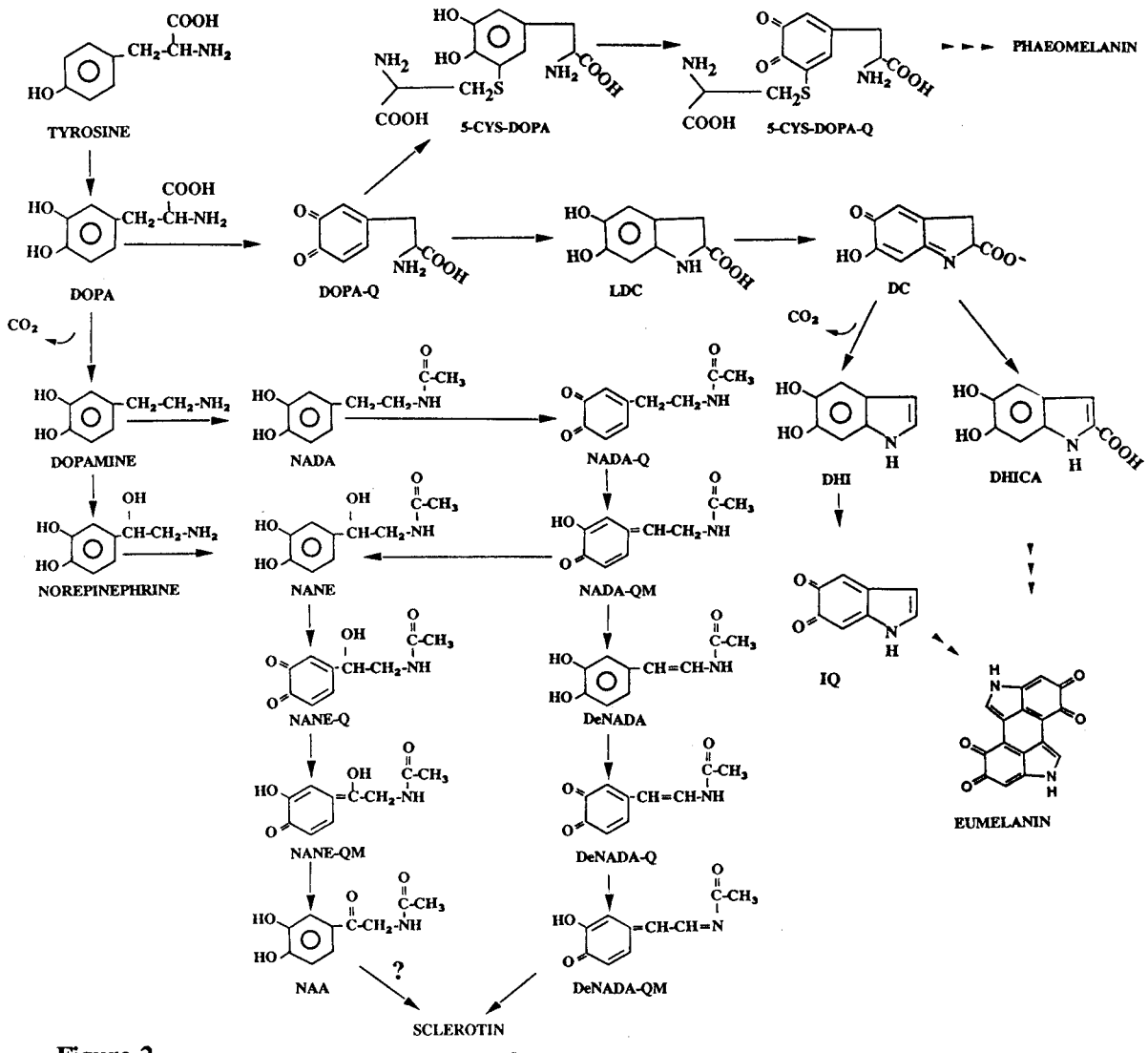
TYROSINE
5-CYS-DOPA
5-CYS-DOPA-Q
PHAEOMELANIN
DOPA
DOPA-Q
LDC
DC
DOPAMINE
NADA
NADA-Q
DHI
DHICA
NOREPINEPHRINE
NANE
NADA-QM
IQ
NANE-Q
DeNADA
EUMELANIN
NANE-QM
DeNADA-Q
NAA
DeNADA-QM
SCLEROTIN

Figure 2.

Figure 3. Major pathways involved in melanogenesis and sclerotization. Note that dopamine, the decarboxylated product of dopa, serves both pathways.

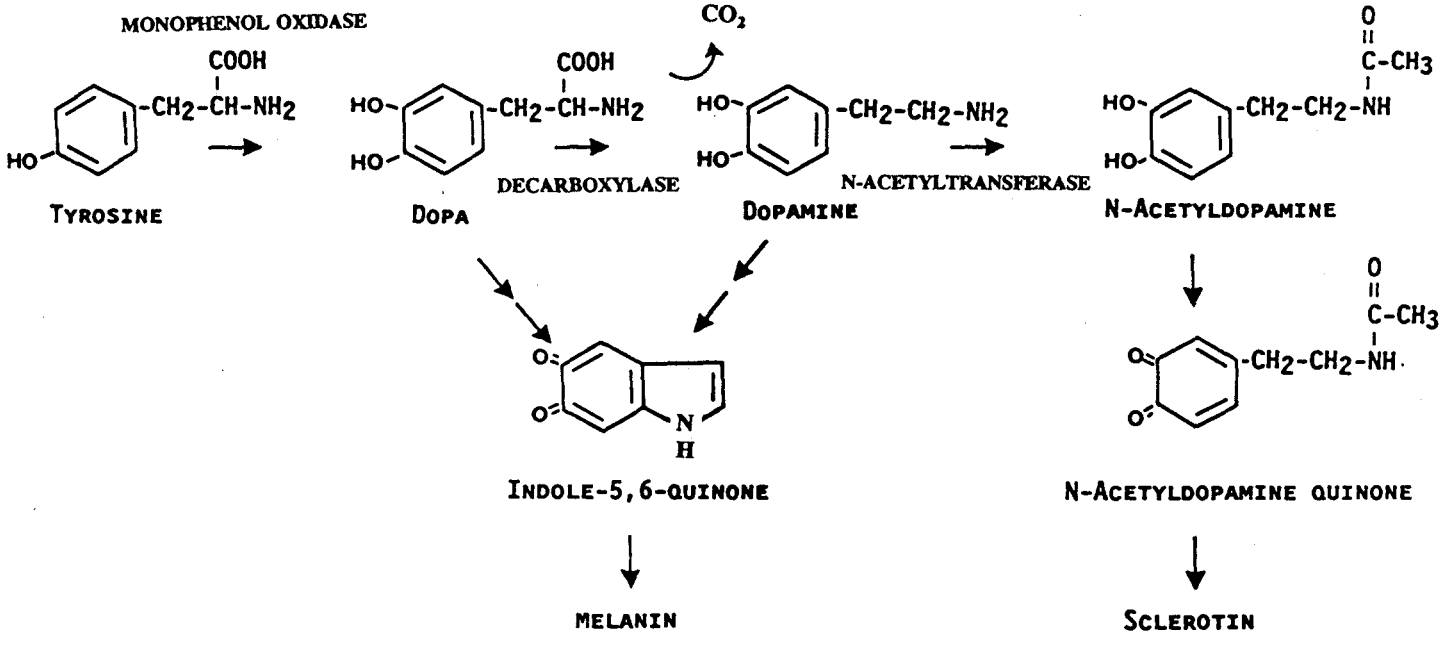
MONOPHENOL OXIDASE
COOH
-CH2-CH-NH2
HO
TYROSINE
CO2
COOH
HO
HO
-CH2-CH-NH2
DECARBOXYLASE
DOPA
HO
HO
-CH2-CH2-NH2
DOPAMINE
N-ACETYLTRANSFERASE
O
C-CH3
HO
HO
-CH2-CH2-NH
N-ACETYLDOPAMINE
O
O
N
H
INDOLE-5,6-QUINONE
MELANIN
O
C-CH3
O
O
-CH2-CH2-NH
N-ACETYLDOPAMINE QUINONE
SCLEROTIN

Figure 3.

Figure 4. Major metabolic pathways involved in the metabolism of tyrosine. Throughout most of larval development tyrosine (as well as dopa) is metabolized by transamination and decarboxylation. During late larval development just prior to the formation of the pupal case the metabolism of tyrosine is switched to form components used to form melanin and sclerotin. (Modified from Sekeris and Fragoulis, 1989).

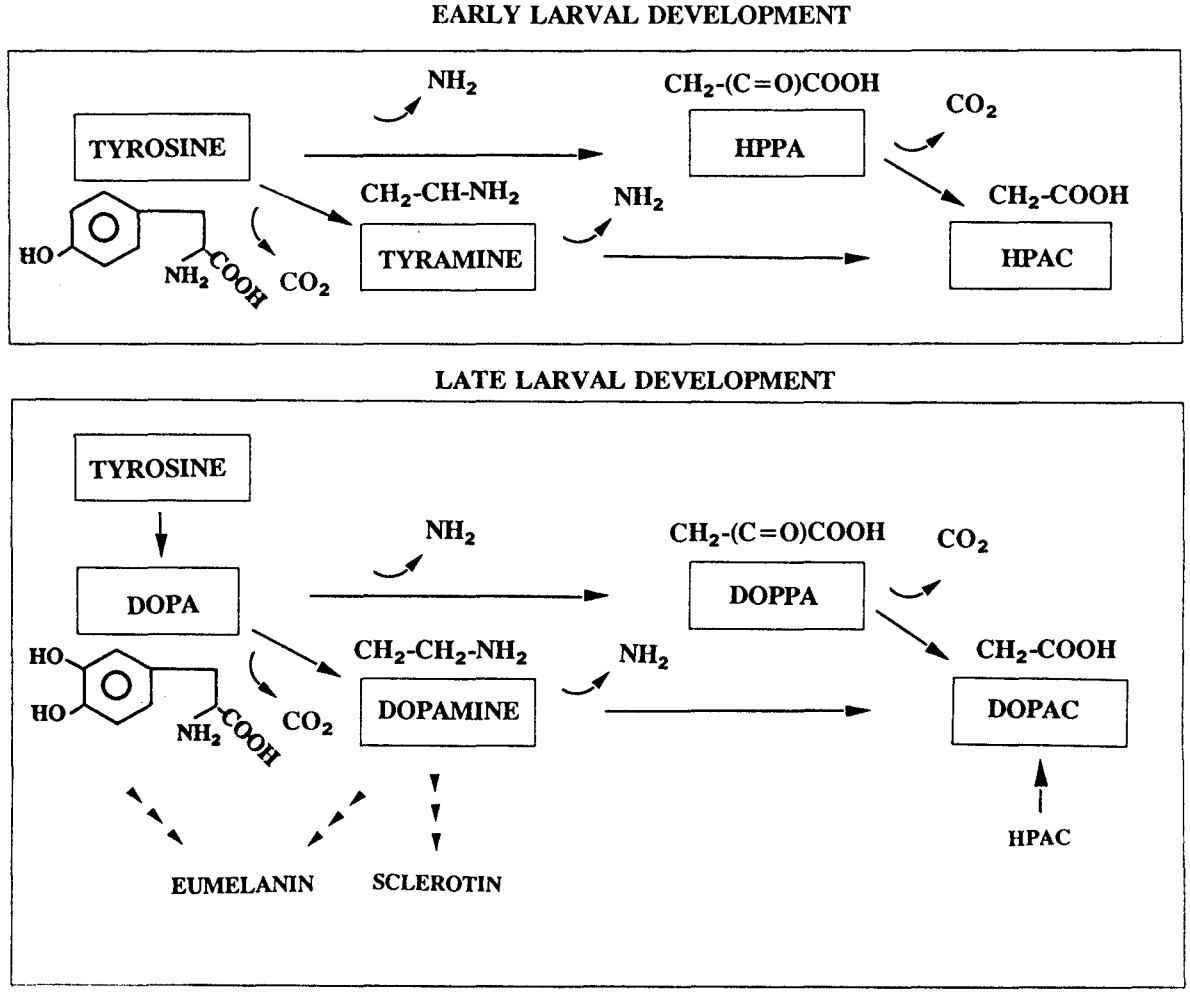
EARLY LARVAL DEVELOPMENT
TYROSINE
NH2
CH2-(C=O)COOH
HPPA
CO2
CH2-COOH
HPAC
CH2-CH-NH2
TYRAMINE
NH2
HO
NH2
COOH
CO2
LATE LARVAL DEVELOPMENT
TYROSINE
DOPA
NH2
CH2-(C=O)COOH
DOPPA
CO2
CH2-COOH
DOPAC
HPAC
CH2-CH2-NH2
DOPAMINE
NH2
HO
HO
NH2
COOH
CO2
EUMELANIN
SCLEROTIN

Figure 4.

Figure 5. Micrographs illustrating melanotic encapsulations made against the parasitic wasp *Leptopilina*. Melanotic capsule is seen in the larva (A), pupa (B), and adult (C) stages of *D. melanogaster*.

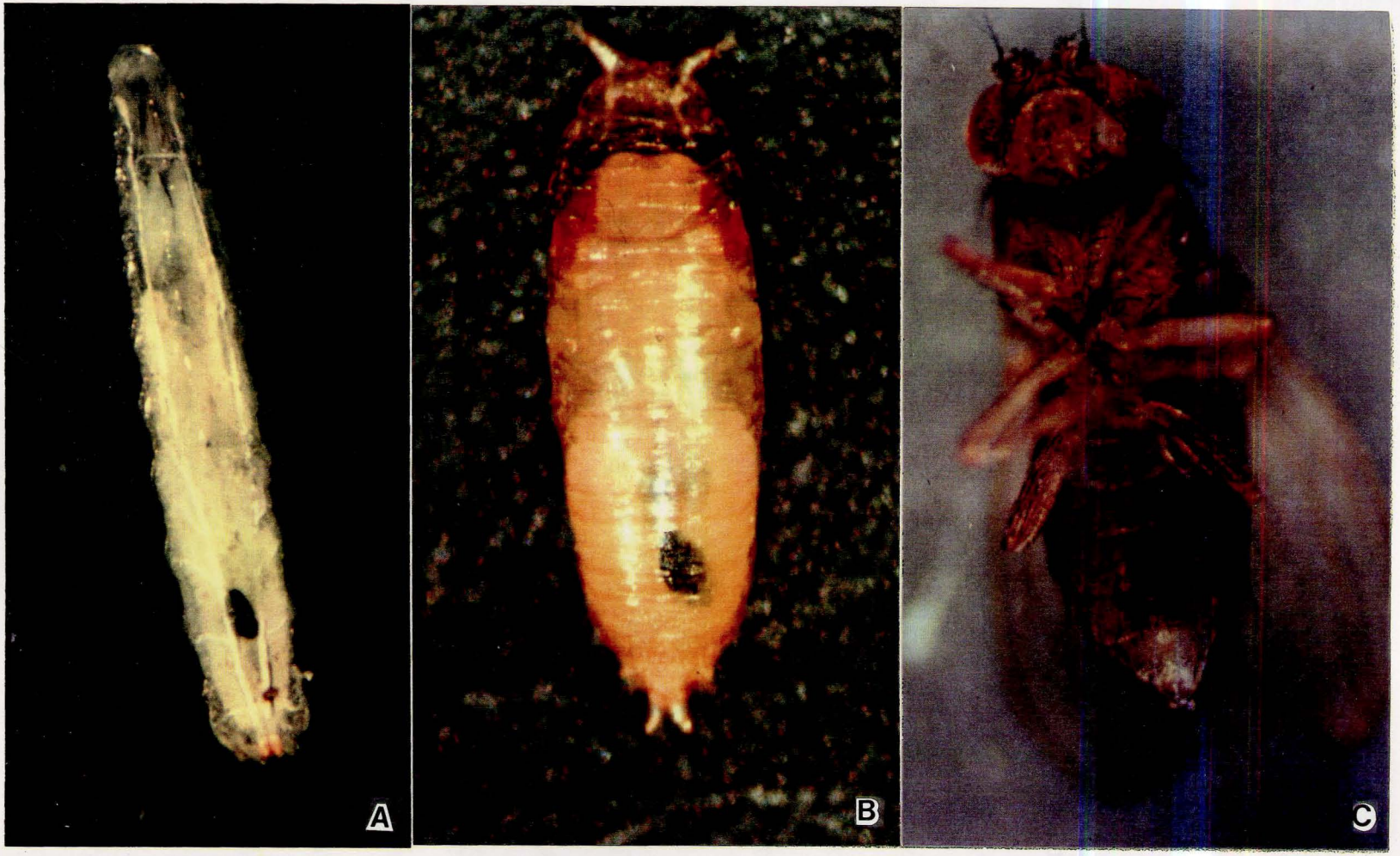
A
B
C

Figure 6. Representative HPLC-ED chromatographic profiles from control (A) and parasitized (B-E) immune reactive larvae at 20 hr post-infection. Elution times are given in minutes. The HPLC-ED system that was used to analyze 5 µl samples of hemolymph consisted of a Bioanalytical Systems (West Lafayette, IN) LC-4B electrochemical detector equipped with a glassy carbon electrode maintained at a potential of + 700 mV versus a Ag/AgCl reference electrode and a sensitivity range of 20 nA. Separations were achieved at 40°C by a BAS Phase-II, 3-µm ODS reverse phase column (3.2 mm I.D. x 10 cm). A Gilson (Madison, WI) 712 HPLC System Controller was used to integrate peak dimensions. The standard mobile solvent system was used and was comprised of 0.1 M citrate buffer (pH 2.9) containing 5% acetonitrile, 0.5 mM sodium octylsulfate, and 0.7 mM Na_2EDTA.

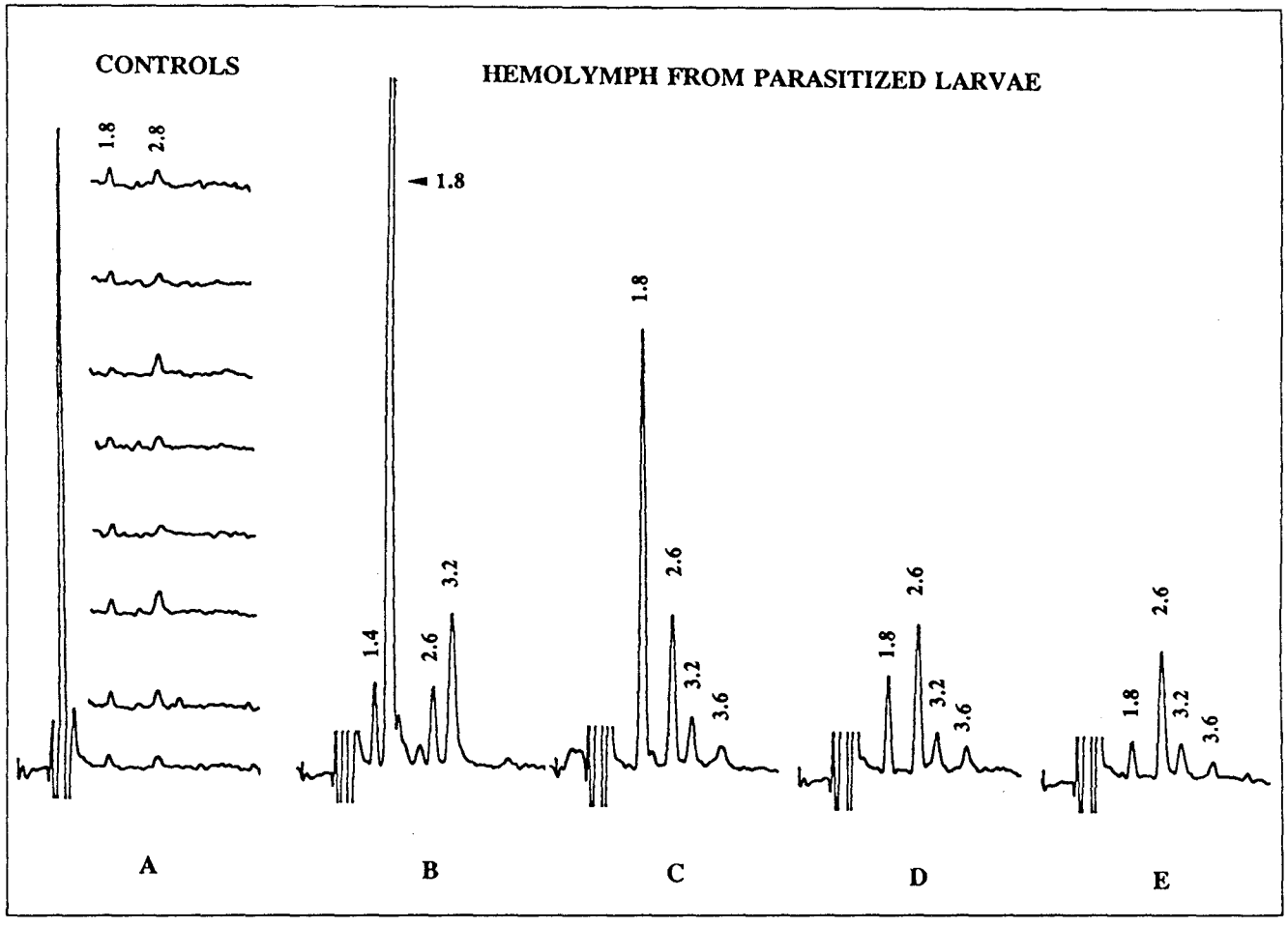
CONTROLS
HEMOLYMPH FROM PARASITIZED LARVAE
1.8
2.8
1.8
1.4
2.6
3.2
1.8
2.6
3.2
3.6
1.8
2.6
3.2
3.6
1.8
2.6
3.2
3.6
A
B
C
D
E

Figure 6.

Figure 7. Representative HPLC-ED chromatograms of hemolymph samples from nonparasitized and parasitized larvae of susceptible (S) and immune reactive (R) strains of *D. melanogaster* 20 h post-infection. Note that the profile of the parasitized, nonreactive S strain is similar to those of nonparasitized controls. The electrochemical assay conditions were similar to those given in Figure 5.

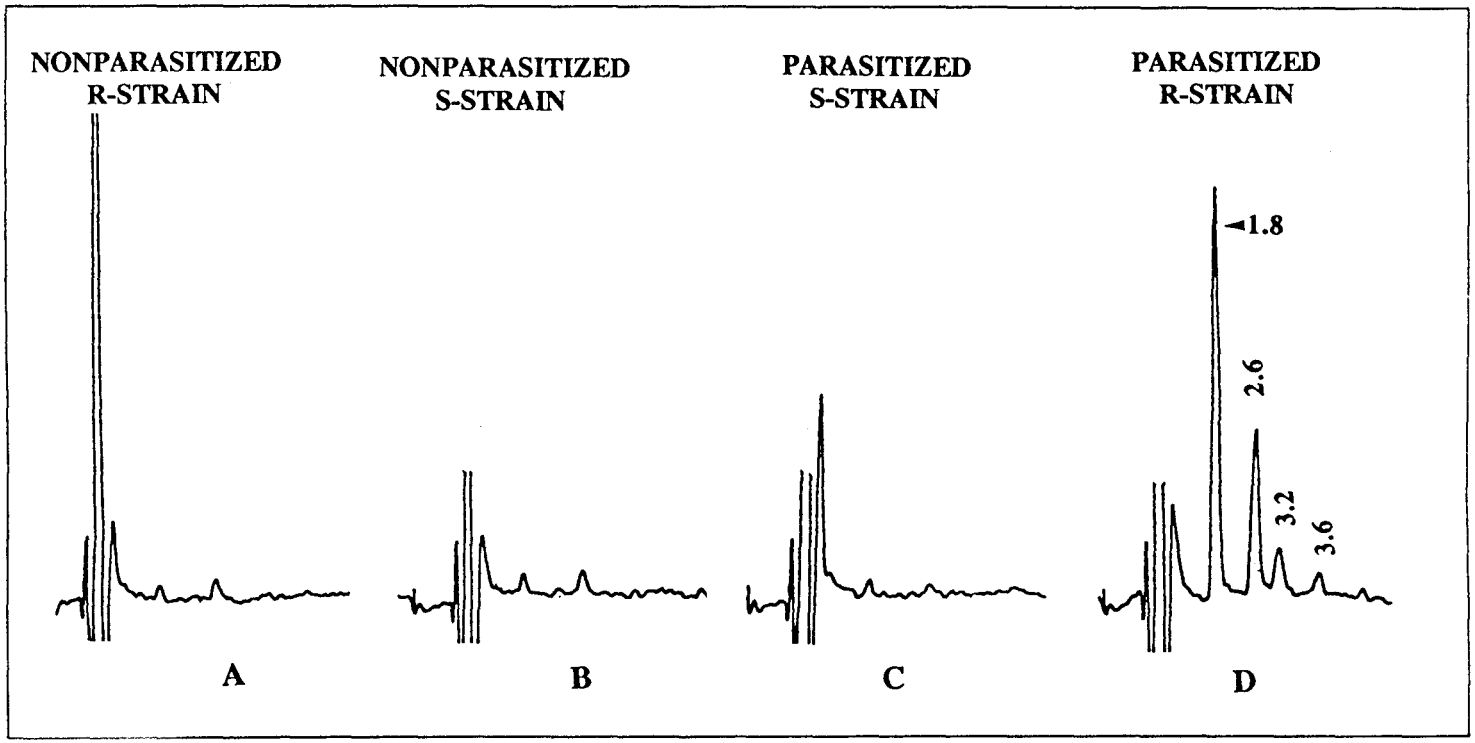
NONPARASITIZED
R-STRAIN
NONPARASITIZED
S-STRAIN
PARASITIZED
S-STRAIN
PARASITIZED
R-STRAIN
1.8
2.6
3.2
3.6
A
B
C
D

Figure 7.

Figure 8. Identification of hemolymph components from immune reactive larvae 20 hr post-infection. (A) Standards. (b) Chromatographic profile of hemolymph from immune reactive larva. The hemolymph sample was co-injected with dopa (C), NAA (D), and DHI (E). Hemolymph components that co-eluted with standards were co-injected and run with three different solvent systems. The electrochemical assay conditions were similar to those given in Figure 5, and a solvent system comprised of 5% acetonitrile and 2.9 pH.

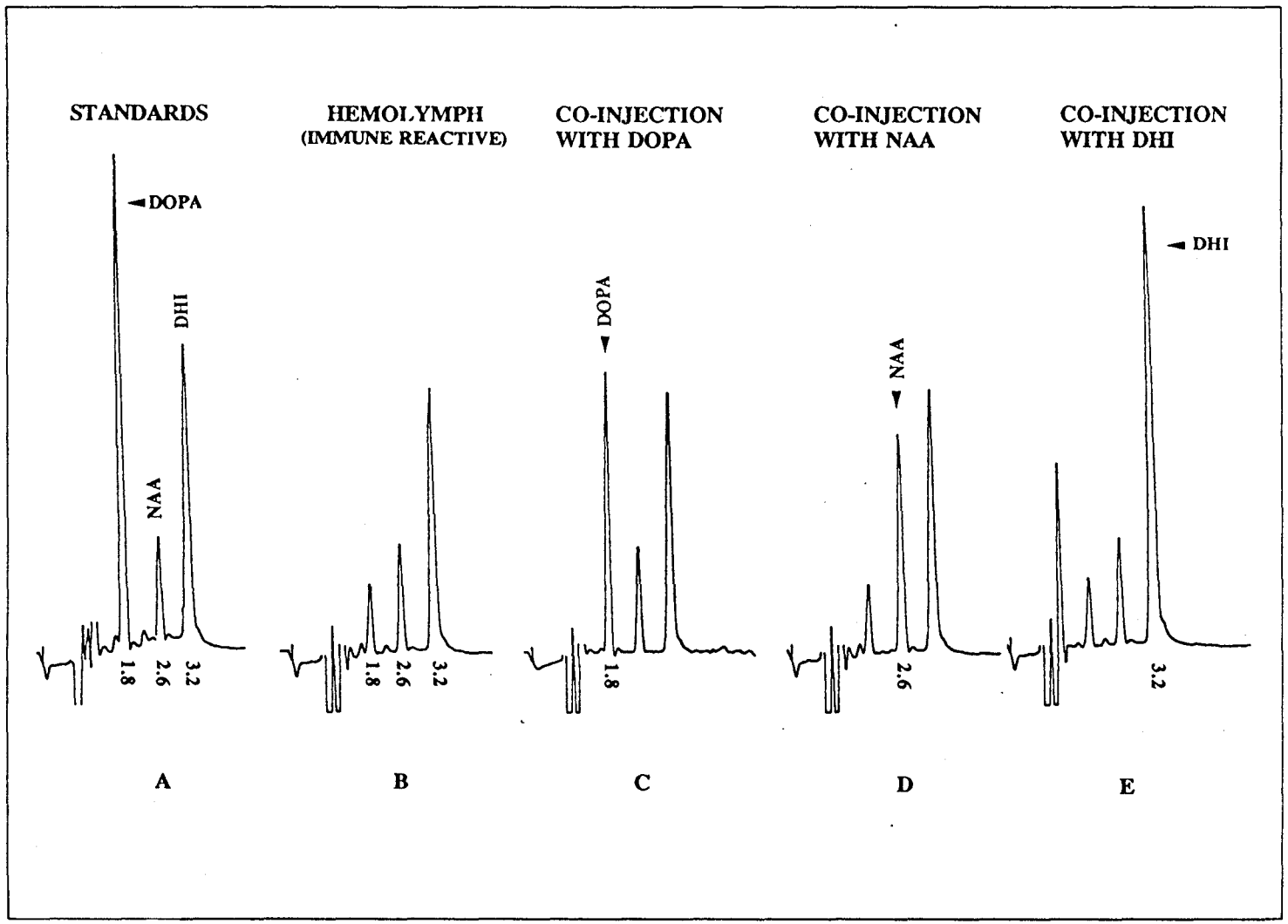
STANDARDS
HEMOLYMPH
(IMMUNE REACTIVE)
CO-INJECTION
WITH DOPA
CO-INJECTION
WITH NAA
CO-INJECTION
WITH DHI
DOPA
NAA
DHI
1.8
2.6
3.2
A
B
C
D
E

Figure 8.

Figure 9. Typical chromatograms of pooled hemolymph samples from nonparasitized and parasitized immune reactive larvae at various intervals post-infection. The electrochemical assay conditions were similar to those given in Figure 5.

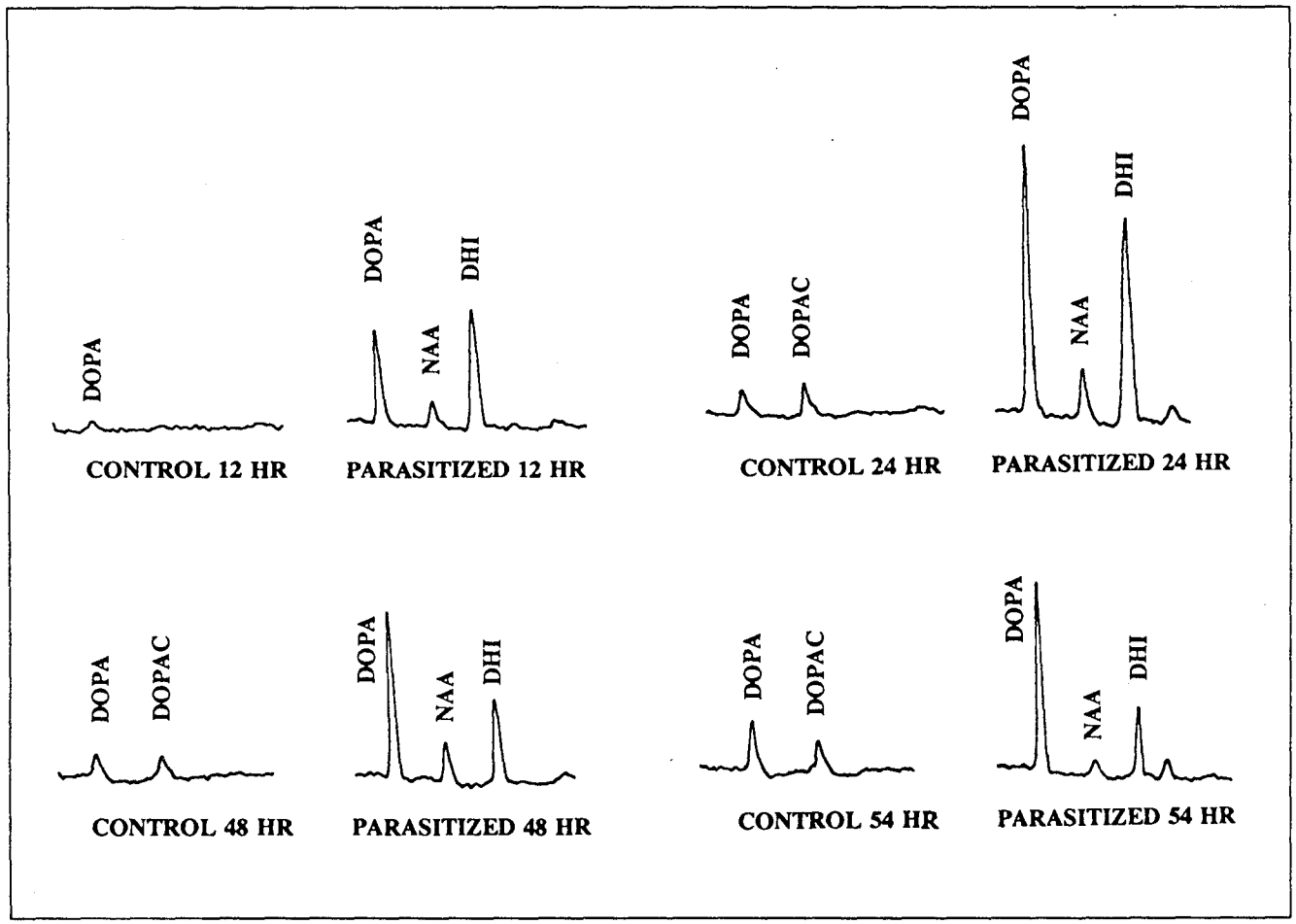
DOPA
CONTROL 12 HR
DOPA
NAA
DHI
PARASITIZED 12 HR
DOPA
DOPAC
CONTROL 24 HR
DOPA
NAA
DHI
PARASITIZED 24 HR
DOPA
DOPAC
CONTROL 48 HR
DOPA
NAA
DHI
PARASITIZED 48 HR
DOPA
DOPAC
CONTROL 54 HR
DOPA
NAA
DHI
PARASITIZED 54 HR

Figure 9.

Figure 10. Typical HPLC-ED chromatographic profiles of hemolymph catecholamines taken from non-parasitized control S strain larvae (A) and parasitized larvae of both S strain (B) and R strain (C and D) at 24 hr post-infection. The biochemical response made by S strain larvae against *A. tabida* (D) was similar to that made by R strain hosts against either *L. boulardi* (B) or *A. tabida* (not shown). The hemolymph catecholamine profile of S strain larvae when parasitized by *L. boulardi* (C) was virtually identical to non-parasitized controls. The elution times of the four electroactive components in the above samples from immune reactive hosts were 1.8, 2,6, 3.2, and 3.6 min. Occasionally, a fifth component was observed with an elution time of 1.4 min. Hemolymph extracted from a small incision in the host cuticle was collected with a microcapillary pipette and placed into 50 µl 0.8 M citric acid stop buffer (pH 2.4). After taking the hemolymph samples each host was dissected and examined to verify the presence of the parasite and the host immune response, if manifested. To obtain individual chromatographic profiles of hemolymph catecholamines, 5 µl of each sample of hemolymph was analyzed by HPLC-ED. The HPLC-ED conditions used are given in Figure 5.

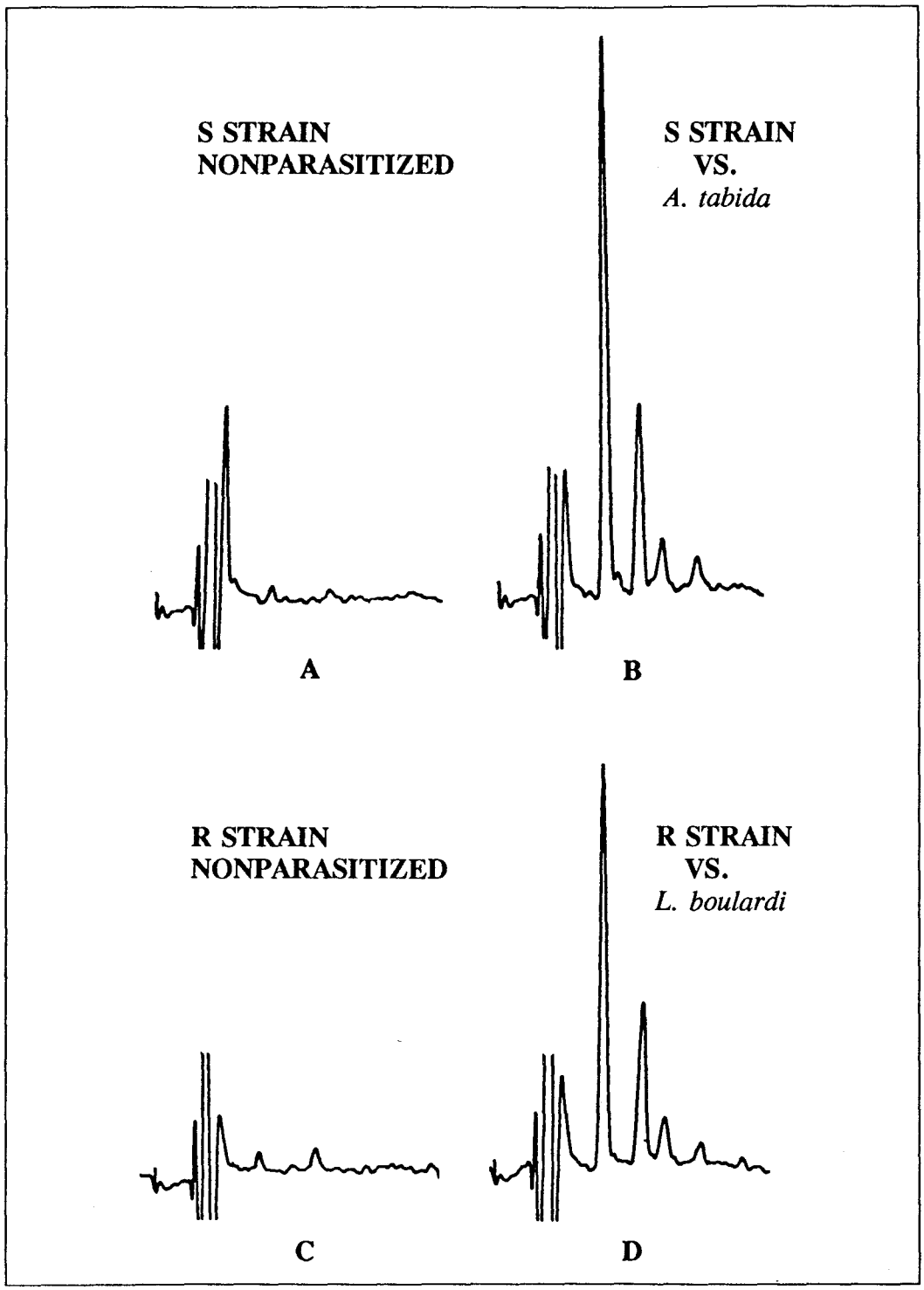
S STRAIN
NONPARASITIZED
S STRAIN
VS.
A. tabida
A
B
R STRAIN
NONPARASITIZED
R STRAIN
VS.
L. boulardi
C
D

Figure 10.

Figure 11. Chromatograms of typical reaction profiles produced in assays for *D. melanogaster* larval monophenol oxidase activity. Rate of enzyme activity was based on product (dopamine) formation over time and concentration of protein. HPLC-Ed conditions were similar to those given in Figure 5, except that the solvent system comprised of 2.5% acetonitrile, and the potential was + 850 mV.

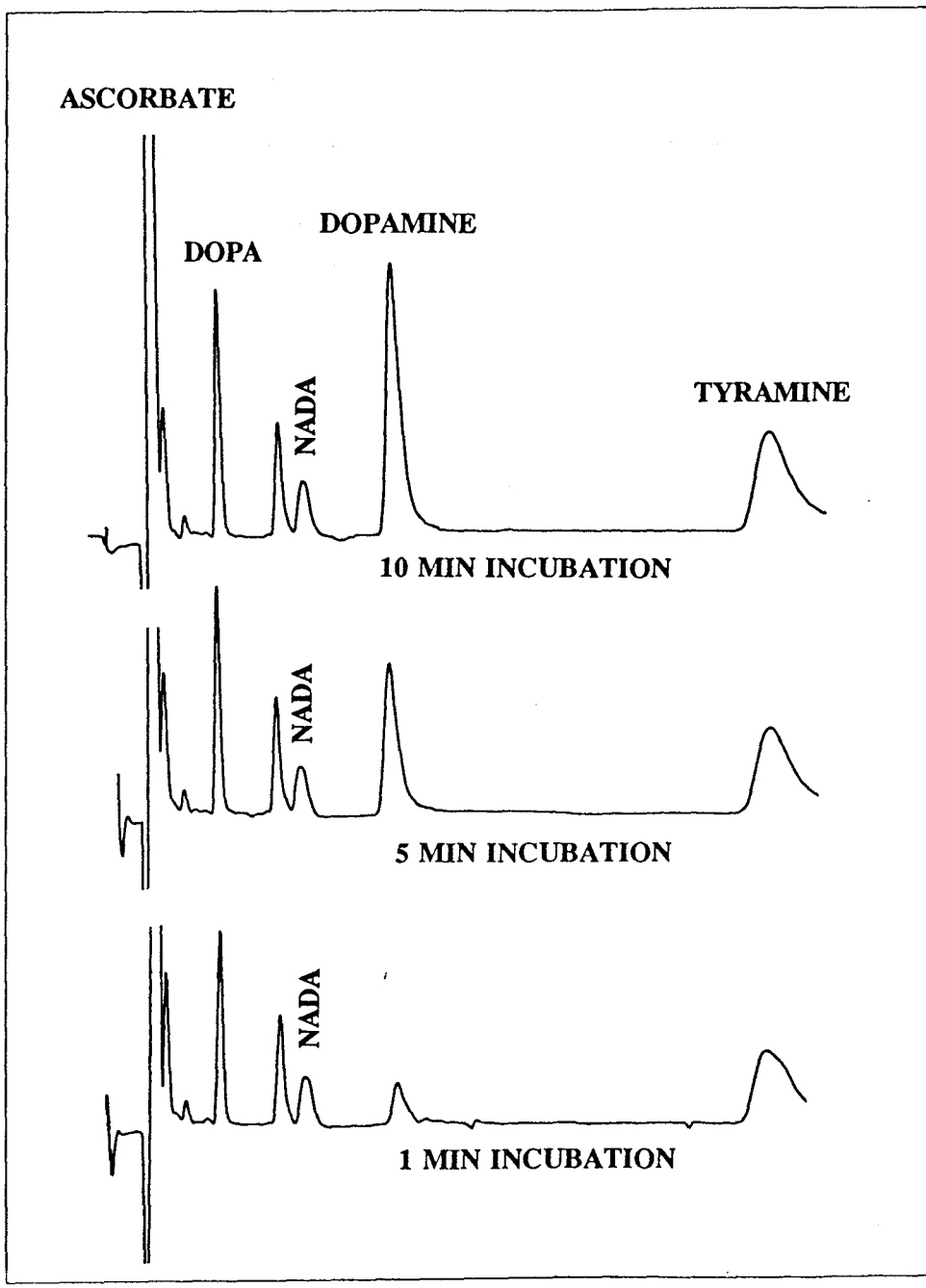
ASCORBATE
DOPA
DOPAMINE
NADA
TYRAMINE
10 MIN INCUBATION
NADA
5 MIN INCUBATION
NADA
1 MIN INCUBATION

Figure 11.

Figure 12. Comparison of monophenol oxidase activity in nonparasitized and parasitized *D. melanogaster* larvae at 24 and 30 hr post-infection by *L. boulardi*. Data represent the means of three tests ($\pm$ S.E.).

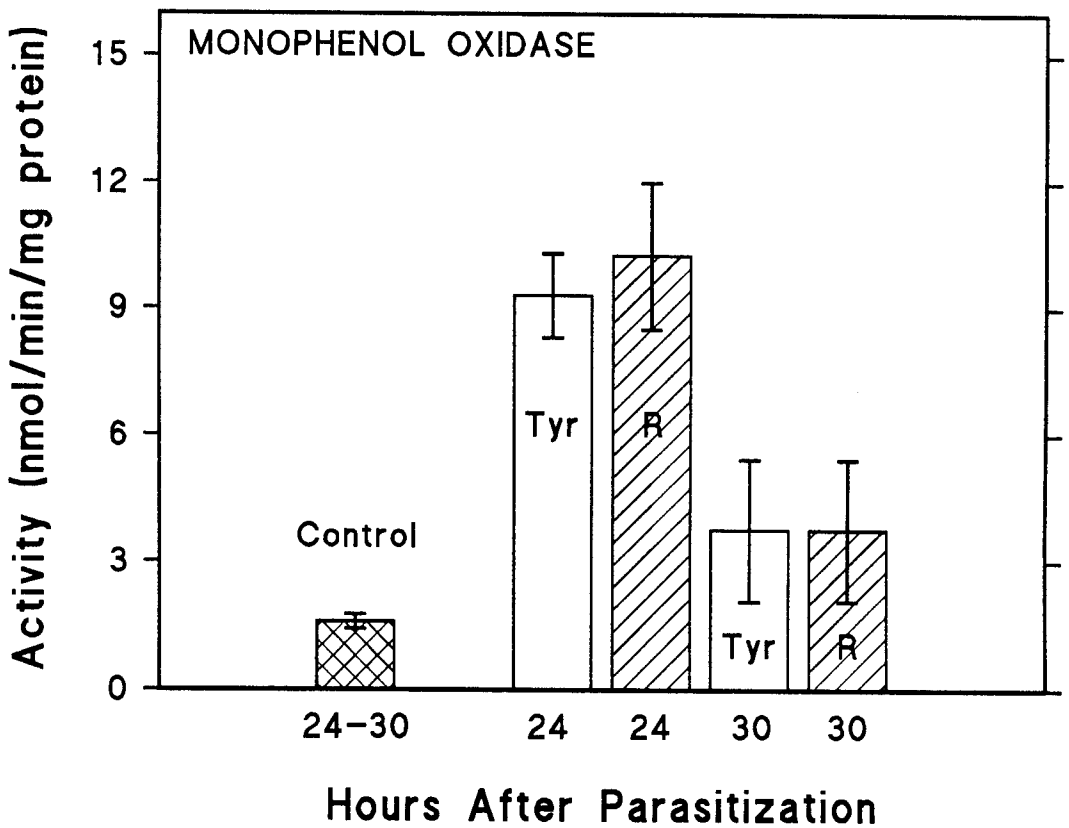
MONOPHENOL OXIDASE
Activity (nmol/min/mg protein)
15
12
9
6
3
0
Control
Tyr
R
Tyr
R
24–30
24
24
30
30
Hours After Parasitization

Figure 12.

Figure 13. (A) Elution times of the substrates dopamine (DA) and norepinephrine (NE) and their reaction products, N-acetyldopamine (NADA) and N-acetylnorepinephrine (NANE), respectively, that were considered for use in assays of N-acetyltransferase activity. (B) Sample of *D. melanogaster* enzyme extract from immune reactive larvae showing DHI eluting at 3.3 min. Since endogenous DHI was not also clearly separated from NADA produced in assays employing DA as the substrate (C), N-acetyltransferase activity was based on the formation of NANE from the substrate NE.

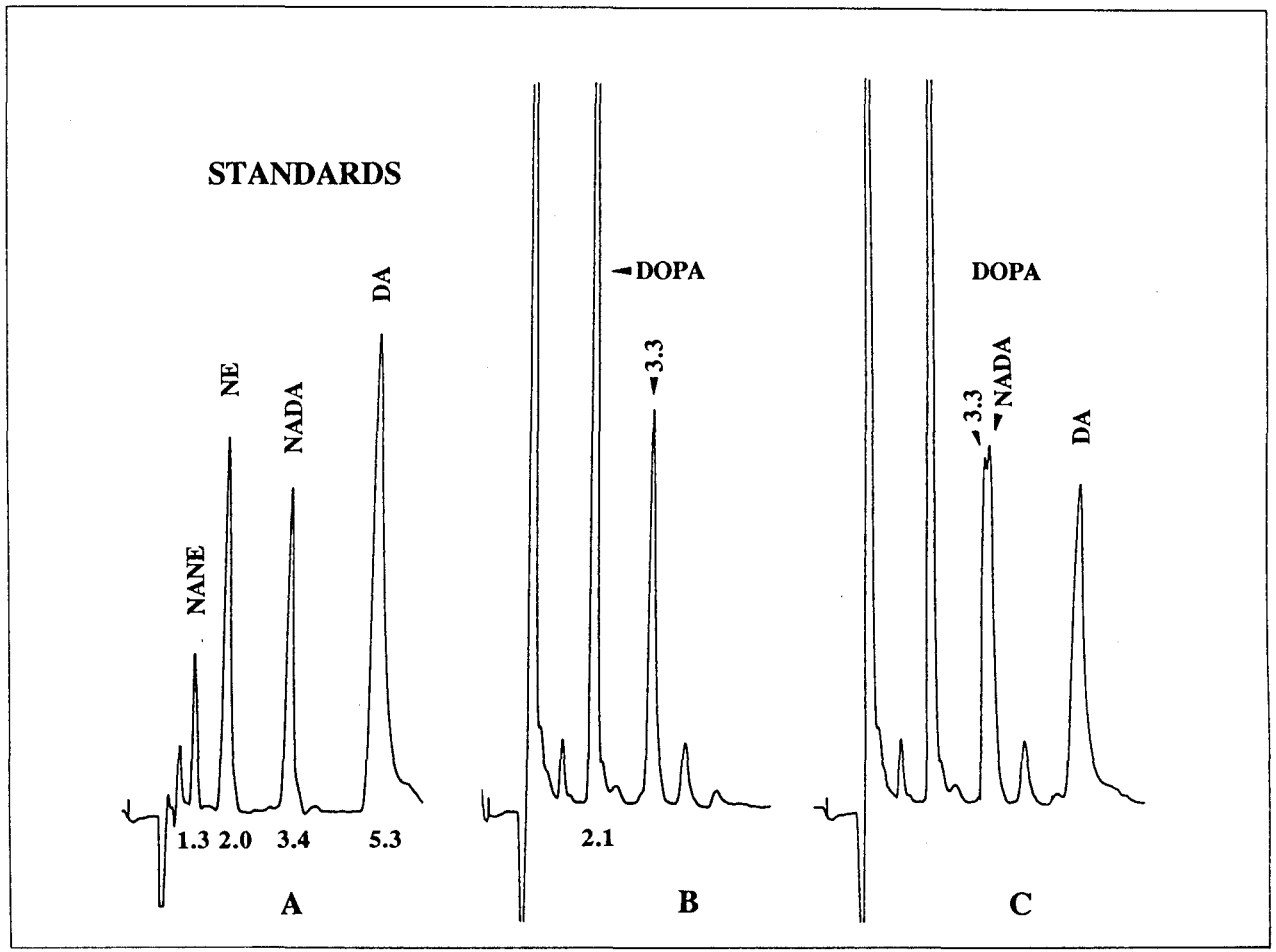
STANDARDS
NANE
NE
NADA
DA
1.3
2.0
3.4
5.3
A
DOPA
3.3
2.1
B
DOPA
3.3
NADA
DA
C

Figure 13.

Figure 14. Chromatograms showing the clear separation of the product NANE formed from the acetylation of NE. The elution times for NANE was 1.4 min and for NE 2.1 min. HPLC-Ed conditions were similar to those given in Figure 10.

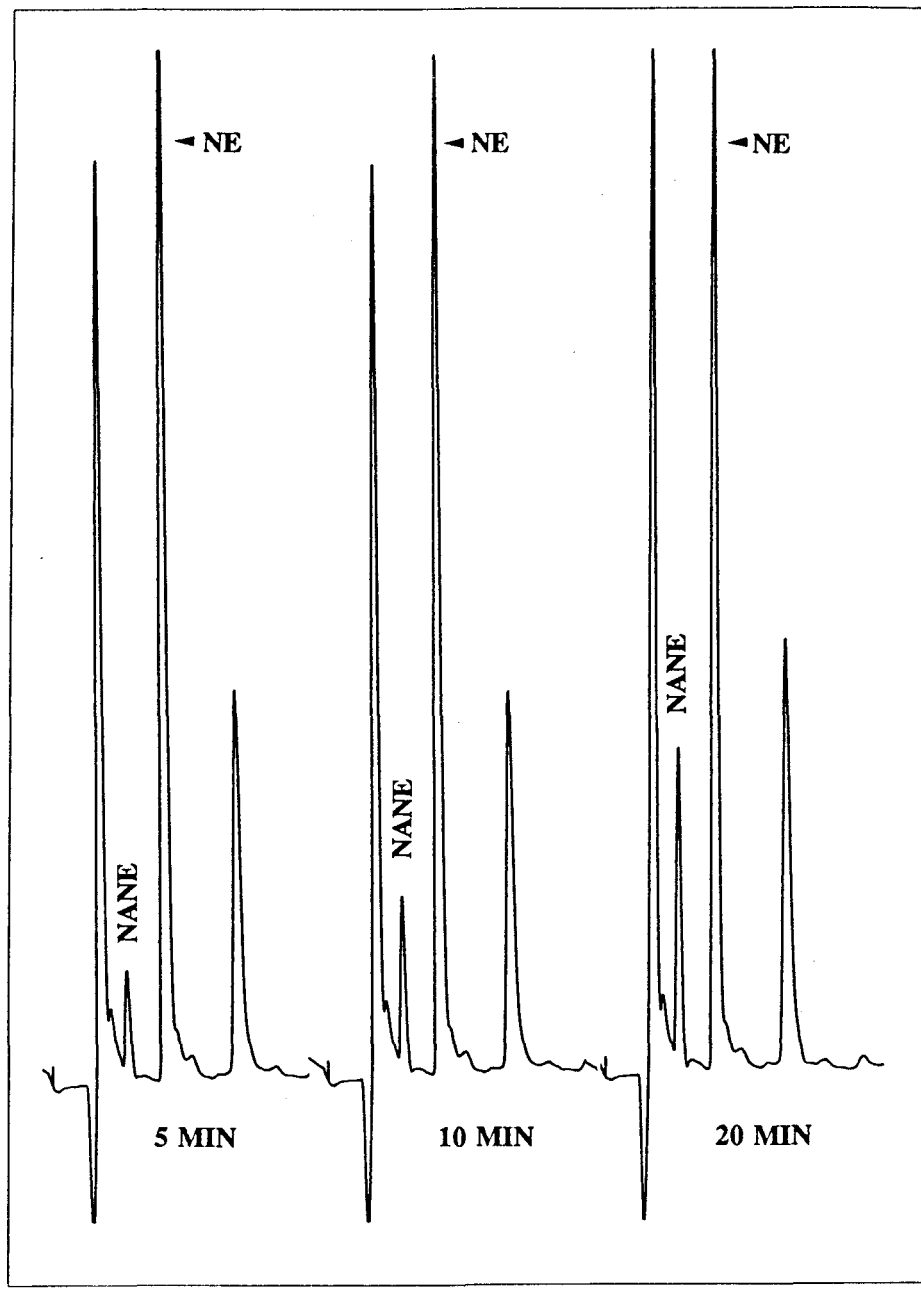

Figure 14.

Figure 15. Comparison of N-acetyltransferase activity in nonparasitized and parasitized *D. melanogaster* larvae at 24 and 30 hr post-infection by *L. boulardi*. Data represent the means of three tests ($\pm$ S.E.).

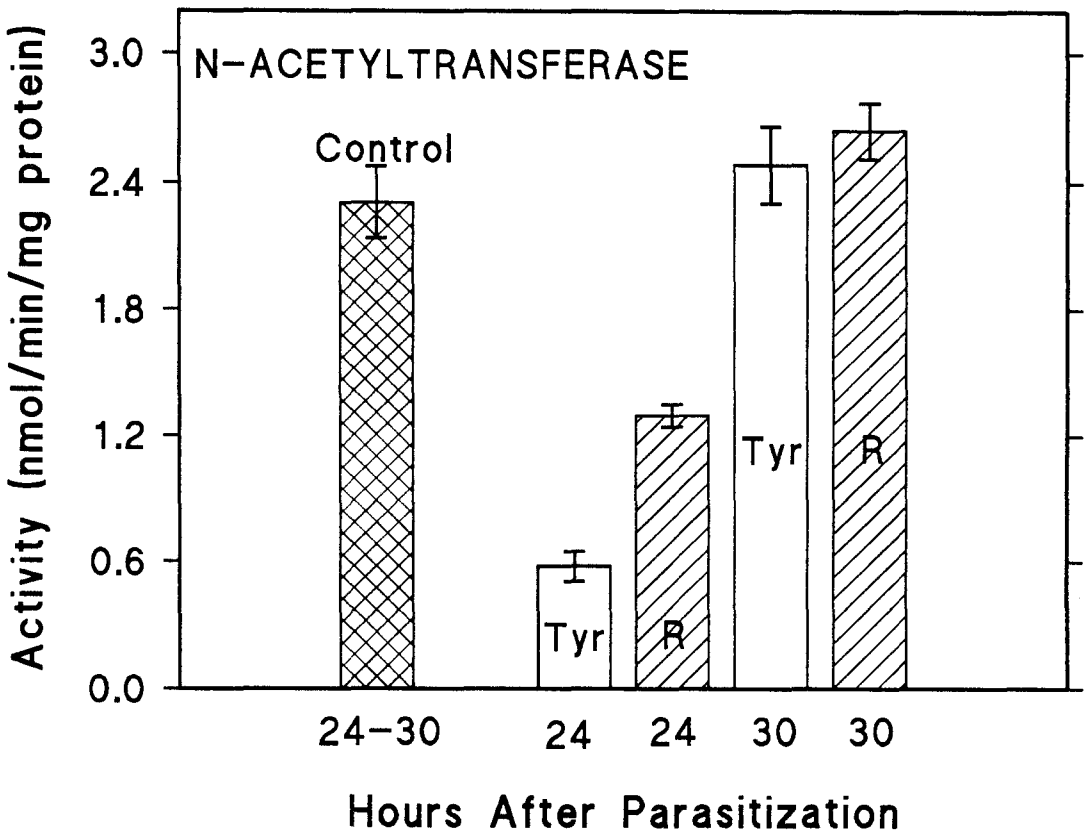
N-ACETYLTRANSFERASE
Activity (nmol/min/mg protein)
3.0
2.4
1.8
1.2
0.6
0.0
Control
Tyr
R
Tyr
R
24–30
24
24
30
30
Hours After Parasitization

Figure 15.

Figure 16. (A) Chromatogram showing the elution times (min) of various standards which were used to identify HPAC in assays for tyrosine aminotransferase activity. (B) Chromatogram of a larval enzyme extract with endogenous dopa label as a reference. (C) Chromatogram showing product formed (elution time 4.7 min) after 10 min incubation. HPLC-Ed conditions were similar to those given in Figure 10.

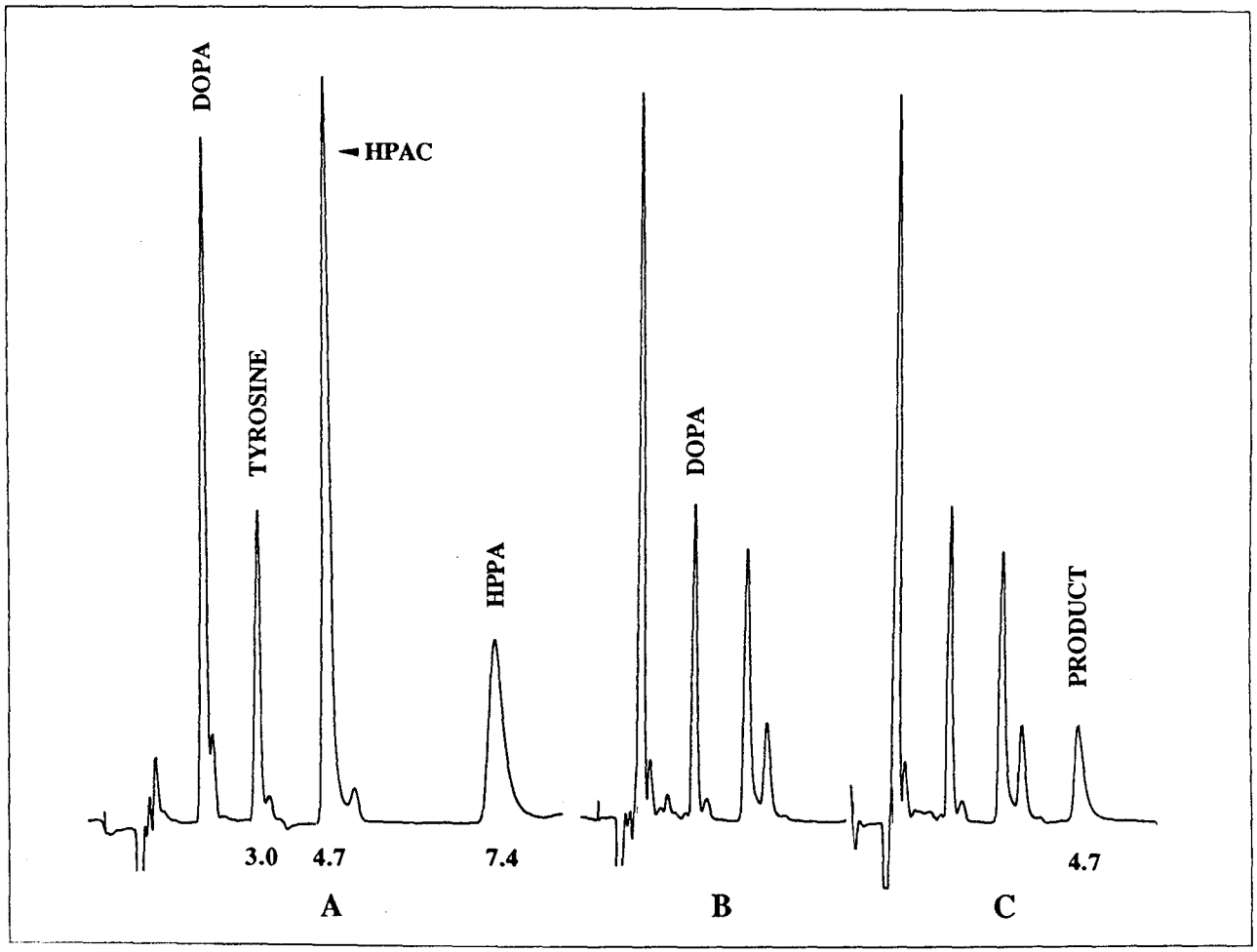

Figure 16.

Figure 17. (A) chromatogram of a 5 µl aliquot of a tyrosine aminotransferase reaction mixture showing product with an elution time of 4.7 min which was identical to the authentic standard HPAC (B). (C) When HPAC was added to an aliquot of the reaction mixture both product and standard co-eluted. HPLC-Ed conditions were similar to those given in Figure 10.

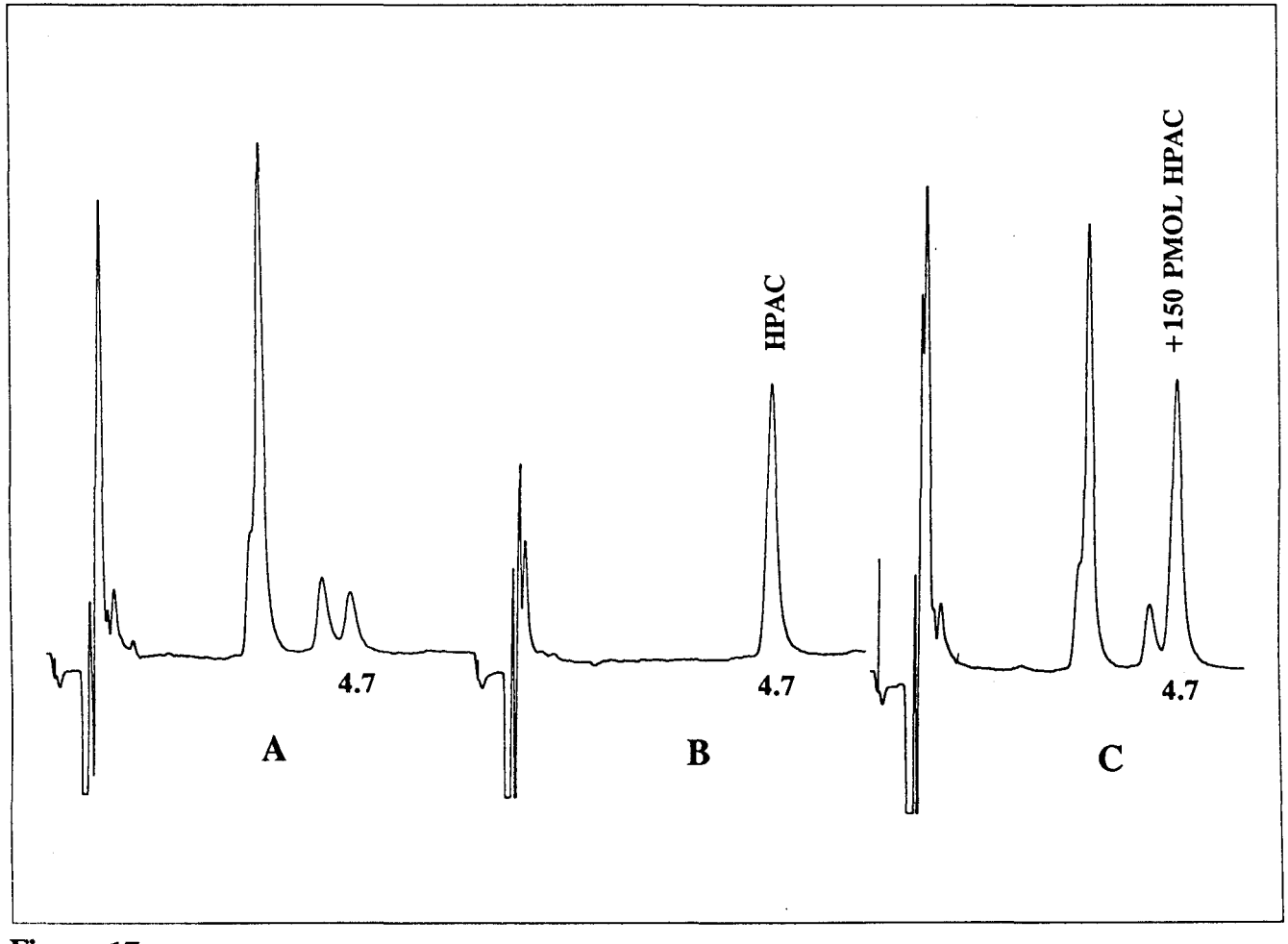

Figure 17.

Figure 18. Comparison of tyrosine aminotransferase activity in nonparasitized and parasitized *D. melanogaster* larvae at 24 and 30 hr post-infection by *L. boulardi*. Data represent the means of three tests (± S.E.).

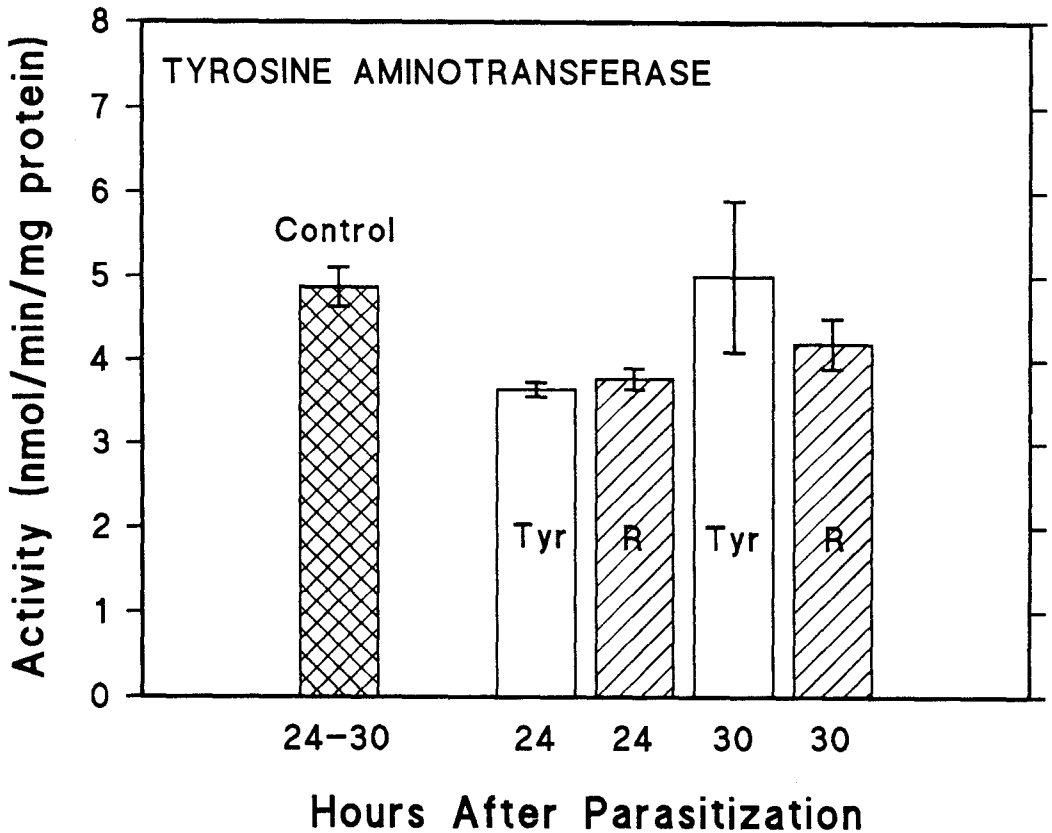
TYROSINE AMINOTRANSFERASE
Activity (nmol/min/mg protein)
8
7
6
5
4
3
2
1
0
Control
Tyr
R
Tyr
R
24–30
24
24
30
30
Hours After Parasitization

Figure 18.

Table 1. Encapsulation Capabilities of Various Host Strains of *D. melanogaster* Against the Parasitic Wasp, *L. boulardi*.

Host strain	No. hosts parasitized	% Encapsulated parasite eggs (+ SE)
R	123	82.6 (3.8)
S	139	3.9 (3.5)
Tyr-1	150	78.3 (1.9)
Ddc [1]	216	80.1 (2.2)
Ddc [2]	212	8.5 (1.4)

All strains were parasitized at 25°C, except the temperature sensitive Ddc mutant which was parasitized at (1) 20°C and (2) 29°C.

Table 2. List of Standards Screened by HPLC-ED for identification of Hemolymph Components in Immune reactive Larvae of *D. melanogaster*

Arterenone
N-Acetylarterenone
N-Acetyldopamine
N-Acetylnorepinephrine
N-Acetylphenylalanine
N-β-Alanyldopamine
Caffeic acid
Deoxyepinephrine
3,4-Dihydroxybenzoic acid
3,4-Dihydroxybenzylamine
2,3-Dihydroxybenzaldehyde
3,4-Dehydroxybenzaldehyde
3,4-Dihydroxymandelic acid
3,4-Dihydroxyphenylacetaldehyde
3,4-Dihydroxyphenylacetic acid
3,4-Dihydroxyphenylglycol
3,4-Dihydroxyphenylethanol
3,4-Dihydroxyphenylglycoaldehyde
3,4-Dihydroxyphenylserine
5,6-Dihydroxyindole
Dopa
Dopamine
Epinephrine
Gentisic Acid
Homovanillic acid
Homogentisic acid
5-Hydroxytryptamine
6-Hydroxydopamine
p-Hydroxybenzoic acid
p-Hydroxyphenylpyruvic acid
p-Hydroxyphenylacetic acid
p-Hydroxymandelic acid
Metanephrine
α-Methyl-dopa
α-Methyl-tyrosine
O-Methyl-dopa
3-Methoxytyramine
3-Methoyxy-4-hydroxyphenylacetaldehyde
3-Methoyxy-4-hydroxyphenylethanol
3-Methoyxy-4-hydroxyphenylglycol
3-Methoyxy-4-hydroxyphenylglycoaldehyde
3-Methoyxy-4-hydroxy
Norepinephrine
Normetanephrine
Octopamine
Tyramine
Tyrosine
Vanillylmandelic acid

Table 3. Immune Capacity of Two Host Strains of *D. melanogaster* Against Two Species of Parasitic Wasp, *L. boulardi* and *A. tabida*. Host age of second instar larvae when parasitized was 50 + 3 hr (25°C). Hosts were dissected at lease 48 hr post infection when immune responses if present, were clearly manifested (n=3).

	Leptopilina boulardi		*Asobara tabida*	
Host strain	No. hosts parasitized	% Encap. parasite eggs (± SE)	No. hosts parasitized	% Encap. parasite eggs (± SE)
R	123	82.6 (3.8)	133	89.7 (2.9)
S	139	2.9 (1.9)	101	95.3 (3.5)

DISCUSSION

When challenged by the wasp, *L. boulardi,* immune reactive larvae of *Drosophila* elicited an immune response characterized by the accumulation of hemocytes and production of melanin around the eggs of the parasite. In this investigation R strain, *Tyr-1*, and Ddc^{ts2} (at the permissive temperature), exhibited this typical cellular immune response. In contrast, the S (susceptible) strain, and Ddc^{ts2} mutant at the restrictive temperature of 29°C (at which DDC activity is reduced) were virtually non-reactive, encapsulating less than 3 % and 9% of the parasites, respectively (Table 1). Although adults of the *Tyr-1* strain have been reported to possess only 10 % of normal phenol oxidase activity (Pentz et al. 1990), the larvae nevertheless exhibited a high frequency of melanotic encapsulation. Thus, the enzyme deficiency of the adult does not influence the immune capacity of the larvae to generate an immune response.

Of interest is the observation that when S strain larvae were parasitized by *A. tabida*, the hosts were found to be highly immune reactive, encapsulating this parasite at a rate of 95%. Thus, the S-strain appears to be immune competent against *A. tabida*, but may lack an adequate immune

recognition system against *L. boulardi*.

Since certain biogenic amines play an important role during larval development, investigations were performed to evaluate the changes in catecholamines that occurred during an immune response. Examination of hemolymph from immune reactive larvae by HPLC-ED revealed up to six electroactive components. Of the four components identified, only two, DHI and NAA were unique to larvae exhibiting melanotic encapsulation within their hemocoels. Immune reactive larvae lacked DOPAC, a substance found in nonparasitized controls and in nonreactive, susceptible hosts.

The identification of DHI and NAA in immune reactive hosts indicates the involvement of two biochemical pathways in *Drosophila* immunity. Verification of DHI establishes the eumelanin pathway in *Drosophila* defense responses, and provides evidence that the dark pigment produced during melanotic encapsulation reactions is indeed eumelanin, a heteropolymer comprised of various o-hydroquinones and o-quinones derived from tyrosine or dopa, and indole monomers derived from DHI. The reaction sequence producing eumelanin includes the intramolecular cyclization and indolization of dopaquinone to form leucodopachrome,

dopachrome, DHI, and 5,6-indolequinone, the final product of which may be conjugated with various proteins (Fig 2).

The finding of NAA lends supporting evidence to the proposed involvement of quinone isomerase in cuticular sclerotization (Sugumaran, 1991), and suggests that the melanized cellular capsule sequestering parasites in immune reactive *Drosophila* larvae is a composite of eumelanin and sclerotin. Following the oxidation of NADA to its quinone (Fig. 2), quinone methide isomerase is thought to convert NADA-Q to N-acetyldopamine quinone methide (NADA-QM), which may subsequently react with water to form NANE that is ultimately transformed into NAA. Other possible pathways to form sclerotin include the acetylation of NE to NANE with ensuing reactions leading to NAA, or NANE may be completely excluded from the process if NADA-QM is instead dehydrolyzed to dehydro-NADA which is then converted into sclerotin (Fig. 18).

Sclerotization is an ongoing process in the larval and pupal stages of development in *Drosophila* that is heightened during periods of molting. In sclerotization reactions catecholamines are incorporated into the cuticle where they are oxidized to quinones which then covalently cross-link the amino groups of proteins and/or chitin to stabilize the cuticle. Interestingly,

in the very early stages of larval development, the major pathway taken by tyrosine is transamination. An important product of the latter process is HPAC, which may be formed principally by one of two major biochemical pathways. Tyrosine may undergo an initial deamination to form HPPA, and then the latter is decarboxylated to HPAC. Alternatatively, tyrosine is first decarboxylated to tyramine followed by its deamination to HPAC (Fig. 4). Similar processes transform dopa into DOPAC, a component found only in the hemolymph of nonparasitized larvae and nonreactive host larvae of the S strain. It has been suggested that DOPAC is an end product of oxidative degradation of excess catecholamines (Kramer et al., 1984), some of which serve as precursors of melanization and sclerotization (Roseland et al., 1985). However, there is some evidence to suggest that DOPAC functions in cross-linking reactions that form cuticle (Andersen, 1974; Barrett, 1984, 1990). Although its specific function and biosynthetic pathway remain to be elucidated, the absence of DOPAC in immune reactive larvae implies that during melanotic encapsulation responses, certain developmental processes associated with catecholamine metabolism are diverted or altered temporarily, to provide the metabolites needed for immunity.

Normally, catecholamines and related o-diphenols derived from

tyrosine are metabolized by the integrated activities of several enzymes including those responsible for o-hydroxylation, decarboxylation, deamination (transamination), and N-acetylation. The roles played by these enzymes in the cellular immune reactions of larvae of *D. melanogaster* were studied during melanotic encapsulation responses against *L. boulardi* and *A. tabida*. In this investigation a ten-fold increase was noted in MPO activity at 24 hr post-infection. This response is considered essential to the sequence of biochemical changes associated with the immune response, since it represents the initial switch in tyrosine metabolism that generates quinonoid precursors of melanin and sclerotin. Concurrently, the activities of both NAT and TAT were significantly lower than those of controls during this period of encapsulation reactions. At 30 hr after parasitization, when melanotic capsules were nearing completion, MPO activity decreased slightly, but was still approximately 5 times higher than normal. At the same time, however, both NAT and TAT activities had returned to levels comparable to those of controls.

The results of this investigation indicate that when larvae of *Drosophila* are traumatized by endoparasites, metabolites common to a variety of metabolic pathways are temporarily diverted, and appropriated to

the more crucial immune response, i.e. the one with a higher priority for survival. To ensure successful melanotic encapsulation of foreign entities, a response mediated initially by tyrosinase, modifications in the activities of certain other catecholamine metabolizing enzymes such as NAT and TAT would appear to be necessary since they metabolize common biogenic precursors. The diminished activity of TAT in immune reactive larvae correlates well with the absence of DOPAC in these hosts, but the low level of NAT was unexpected. It was anticipated that NAT activity would be augmented during this period to provide for the sclerotization of the capsule components around the parasite. The reduced activity of N-acetyltransferase suggests that the enzyme is not involved in the process of melanotic encapsulation at least not during this stage of infection (i.e., 24 - 30 hr post-infection), or sclerotization of the capsule results from some other biosynthetic pathway, perhaps involving N-β-alanyltransferase, an enzyme that produces other known sclerotin precursors such as N-β-alanyl catecholamines. These results do not preclude the involvement of NAT at a different stage in the response, since enzyme activity was not studied beyond 30 hr post-infection. Once the encapsulation response is complete, normal enzymatic activities resume and development continues.

Data from the temperature sensitive Ddc^{ts2} mutant suggests that the enzyme DDC also plays a significant role in the development of the melanotic response against endoparasites. Ddc^{ts2} mutants when lacking normal levels of DDC at the restrictive temperature, had a diminished ability to form cellular melanotic capsules around eggs of *Leptopilina*. Since the restrictive temperature reduces the level of DDC activity in Ddc^{ts2} (Wright et al. 1981. Tempel et al. 1984), it seems reasonable to propose that the decrease in immune competence that occurs in the mutant is at least partially associated with the genetic defect (Nappi et al., 1992). As a result of decarboxylation, dopa is converted to DA. Although this catecholamine has long been known to be an important precursor of sclerotin, and more recently an important precursor of eumelanin (Hiruma and Riddiford, 1988), the results of this study suggests that the melanin precursors derived from DA may be more important in forming capsule components than those derived from the oxidation of dopa (see Fig. 3).

It is of interest to note that immune reactive R and S strain larvae of *Drosophila* when parasitized by *L. boulardi* and *A. tabida*, respectively, showed similar cellular (Nappi, 1981) and biochemical mechanisms of defense. It is not known why the S strain is nonreactive against *L. boulardi*.

It is known that when endoparasitic wasps deposit their eggs into their hosts, secretory glands associated with their reproductive passages release a venom that contains polydnavirus particles (see review by Schmidt and Theopold, 1991) into the host hemocoel along with the egg. These substances are believed to interfere with the immune response of the host. The polydnavirus particles coating the egg are thought to protect it from the host's immune reactions (Stoltz and Vinson 1979; Vinson, 1990; Bedwin, 1979; Edson et al. 1981; Fedderson et al. 1986; Rizki and Rizki, 1990; Strand and Noda, 1991). In some cases the particles secreted by endoparasitic wasps were found to be comprised of proteins antigenically related to components of the host's immune system (Fedderson et al. 1986; Webb and Summers, 1990; Berg et al. 1988; Schmidt and Theopold, 1991). This suggests that the protection afforded some parasites is due to the presence of host-like proteins on the parasite egg surface that prevent recognition of molecular similarity to self components. However, in the case of *L. boulardi*, it is unlikely that protection against encapsulation by S strain hosts results from molecular mimicry, since the parasite with the same molecular properties is readily encapsulated in R strains hosts. Another possible explanation is that the immune systems of the R strain hosts may

be able to combat and destroy the polydnavirus particles that would otherwise prevent encapsulation reactions, whereas the S strain hosts may be unable to effectively confront or contain these polydnavirus particles. This allows them to multiply, and in the process incapacitate the host immune system. Data from this study would appear to provide circumstantial evidence to support the hypothesis of parasite suppression, but this finding is amenable to at least one alternative explanation which considers different gene loci for recognition of different parasites. It is possible that S strain hosts, although highly immune competent against *A. tabida*, lack the ability to recognize the Brazzaville strain of *L. boulardi*. Apparently, the selection process operating in the evolution of immune competence as evidenced by R strain hosts promoted their ability to recognize *L. boulardi* and to circumvent the coevolved mechanism of immune suppression possessed by *Leptopilina*.

This study has documented certain alterations in the activities of various catecholamine-metabolizing enzymes during immune reactions, and it is questioned what effect these changes might have on the development of the host. When *Drosophila* larvae terminate feeding in preparation for pupariation, their nutritional resources for continual successful development

become limited. This investigation has shown that in response to parasitic infection, critically important biochemical processes are interrupted and alterations made to divert some of these limited resources away from normal processes to those required for the immune response. Some of these substances are involved in the normal development of the cuticle. Thus, some potential costs incurred by immune competent hosts may include reduced body size, a thinner cuticle, and other altered physiological and/or developmental processes which depend on normal levels of these metabolites and on the temporal regulation of the activity of requisite enzymes. Recent studies show that *D. melanogaster* adults that survived infection by *L. boulardi* by encapsulating parasites are smaller. Females produce fewer eggs (Carton and David, 1983), and males are less efficient at courtship and mating than flies that have developed from uninfected larvae (Bijlsma and Trapman, 1989). However, surviving infection and producing fewer than normal number of offspring is better than not surviving to produce any progeny at all.

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VITA

Emily Vass was born in Chicago, Illinois, on February 11, 1967. In August 1985 she entered Loyola University of Chicago and received a Bachelor of Science degree with a major in Biology in May 1989. She returned to Loyola University in August of 1990 to undertake graduate work in Biology. She was awarded an assistantship in the Department of Biology which facilitated her work and enabled her to complete the Master of Science degree in 1992.

APPROVAL SHEET

The thesis submitted by Ms. Emily Vass has been read and approved by the following committee members, all or Loyola University of Chicago:

Dr. Anthony J. Nappi, Director
Professor, Department of Biology

Dr. Albert J. Rotermund
Associate Professor, Department of Biology

Dr. Frederick H. Wezeman
Professor and Chairperson, Department of Biology

The final copies have been examined by the director of the thesis committee. The signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

15 April 1992

Date

Director's Signature