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The Biochemistry of Melanin Formation in Shrimp.

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IN SHRIMP

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ABSTRACT

Physical and chemical properties of the enzyme(s) which cause black spots in shrimp were studied. Several of these properties were similar to those of phenol oxidases (phenolases) from plant and animal sources: the shrimp enzyme system catalyzed the oxidation of both mono- and o-dihydric phenols; shrimp catecholase required copper for its activity; substances known to inhibit phenolase activity also inhibited activity of shrimp catecholase, these included copper and phenol binding agents, reducing agents and carboxylic acids; the shrimp enzyme(s) appeared to be a water-soluble globulin and could be concentrated by methods similar to those used in the purification of mushroom catecholase; catecholase activity in crude preparations from shrimp was slightly increased by heating at 50°C, for 1 and 2 minutes and energy of activation values compared favorably with those obtained for phenolases from other sources.

Chromatographic and spectrophotometric methods were used to identify phenolic compounds associated with melanin formation. L-tyrosine, L-dihydroxyphenylalanine (dopa) and 2-carboxy-2, 3-dihydroindole-5, 6-quinone (dopachrome) were identified in shrimp samples and the various melanogenic phases indentified with "dopa
melanin" formation observed during the darkening of these samples. Data is presented which suggested that shrimp phenolase is stereospecific for the catalysis of L-dopa. Dialyzates of shrimp blood and press juice continued to darken even at low temperatures (3°C) suggesting that proteins containing phenolic groups may also act as substrates for shrimp phenolase.

Blood fractions from Peneaus setiferus and Peneaus aztecus exhibited high o-dihydric phenolase activities. The "sediment" obtained by centrifuging blood from these shrimp species contained a more highly active o-dihydric phenolase than did "plasma" or "serum" from the same blood. Leucocytes in this blood may contain active phenolase(s) released when shrimp blood is exposed to air. Other evidence presented favors the existence of highly active phenolase(s) in shrimp blood cells. Data was obtained which indicated that hemocyanin could catalyze the oxidation of pyrocatechol, and consequently may be involved in black spot formation in shrimp.

Michaelis constants were calculated for shrimp catecholase and DL-dopa oxidase.
CHAPTER I

INTRODUCTION

When headless shrimp are refrigerated, the abdomen or "tail" develops black spots or bands, usually at the base of the shell segments or across the back of the tail where the shell segments overlap. In general, the discoloration first develops in the membrane which separates the tail muscle from the shell, especially in those areas where the membrane connects the two ends of overlapping shell segments. During refrigerated or frozen storage of whole shrimp, black spot formation usually starts in the cephalothorax, so that the carapace appears black. This is followed by discoloration of the antennae and rostrum or "spine" and the uropod or "tail fin". The crawling legs (periopods) and swimming legs (pleopods) change color first at the joints and this change finally progresses over the entire leg. When the condition becomes severe, the interior of the head becomes a black, soft or mushy mass and the carapace becomes flexible instead of retaining its normal stiff consistency.

In 1951, Fieger (1) concluded that black spot formation in shrimp resulted from the action of tyrosinase or some similar
enzyme. He also showed that numerous inhibitors of tyrosinase such as sodium cyanide, sodium azide, thiourea, cysteine and glutathione also prevented black spot formation. Since tyrosinase catalyzes the oxidation of naturally occurring phenolic compounds to materials which lead to the eventual formation of the black pigments (melanins), he suggested that black spot or black head be referred to as melanotic shrimp, the chemical changes involved be referred to as melanogenesis, and the black coloration as melanosis (2). Subsequently, Alford and Fieger (3) showed that bacterial action was not directly involved in black spot formation and also discounted direct involvement of other microorganisms. In 1954, Faulkner et al. (4) contributed further evidence that black spots formed in shrimp were melanotic in nature.

The experiments reported here represent a quantitative study of the reaction systems involved during black spot formation in shrimp and were designed with the following major objectives in mind:

(a) to study some of the physico-chemical properties of the enzymes involved in black spot formation and to compare these properties with those of phenolases from other sources;

(b) to verify the hypothesis that black spot formation in shrimp during storage is in fact a melanosis derived from oxidation of phenolic derivatives catalyzed by one or more active phenolases;

(c) to determine the anatomical location of such enzymes and their substrates.
CHAPTER II

ENZYMES INVOLVED IN MELANIN FORMATION

Oxidative enzymes from a large variety of sources catalyze reactions leading to melanin formation, and some of these have been thoroughly studied and their properties outlined in comprehensive reviews (5-11). Enzymes of this type are generally referred to as tyrosinases or phenol oxidases (phenolases) although individually they are difficult to classify since many of them catalyze the oxidation of several different phenolic compounds.

Crude preparations of phenol oxidases usually may be distinguished from other oxidases through their ability to catalyze two essentially different oxidations: the insertion of an hydroxyl group into monophenols ortho to the one already present (oxygen transferase activity), and the oxidation of o-dihydric phenols to their corresponding o-quinones. (12). Most impure preparations have both activities in the presence of trace quantities of o-dihydric phenols. The question of whether the same or different enzymes catalyze the oxidation of monophenols and dihydric phenols has stimulated much research and speculation but remains unsettled.
In cases where the enzyme has been sufficiently purified, homogeneity studies by electrophoretic and sedimentation methods indicate that only one protein moiety is implicated in both types of oxidations catalyzed by phenolase(s) prepared from the common edible mushroom *Psalliotia campestris* (13). Lerner et al. (14) also ascribed both tyrosinase (monophenolase) and dopa oxidase (o-dihydric phenolase) activity of certain mammalian enzymes to a single protein entity. No evidence is available to indicate whether or not the mono- and o-dihydric phenolase activities of enzymes from other sources is due to a single protein moiety or to two or more protein moieties. In most instances, purification methods have yielded insufficient quantities of enzyme for electrophoretic, sedimentation and solubility studies necessary for decisive experiments on this problem.

Dawson and Tarpley (9) conveniently classify phenol oxidases from mushroom into monophenolase (cresolase) and o-dihydric phenolase (catecholase) and used p-cresol and pyrocatechol respectively to determine these activities. These substances are not only more soluble but are also more readily oxidized by phenolases than phenolic amines such as tyrosine and 3,4-dihydroxyphenyl alanine in the presence of most phenolases. Other investigators (7&15) refer to enzymes of this type simply as tyrosinase or dopa oxidase depending on the substrate specificity and this may differ for enzymes from various sources.
Phenol-oxidizing enzymes are widespread in nature, especially in plants and invertebrates, but the most thoroughly investigated sources have been the potato and the common mushroom *Psalliota campestris*. Dawson and Tarpley (9) list many references concerning enzymes from both plants and animals but the most pertinent of these in regard to the phenol oxidizing enzymes in shrimp are those of Pinhey (16), Dennell (17), and Bhagvat and Richter (18) who studied these enzymes in Crustacea and other Arthropoda.

Bhagvat and Richter (18) found evidence for the presence of phenol oxidases in the blood of several arthropods, among which were *Odonestris potatoria* (larval), *Bombyx quercus* (larval), *Locusta migratoria* (adult), *Cancer pagurus* L. (adult), and *Homarus vulgaris* (adult). Pinhey (16) reported the presence of tyrosinase in the blood of certain crustaceans including the spider crab *Maia aquinado* and the edible crab *Cancer pagurus*. She showed that the phenol oxidase in the Cancer crab occurred mainly in the leucocytes, and pointed out that these could possibly disintegrate to release enzyme which reacts with substrate present in the serum. Other marine sources of phenol oxidases include octopus blood (19) and cuttlefish sac (20).

Phenol oxidase activity was demonstrated (18) for crystalline hemocyanin from blood of *Cancer pagurus* and catalyzed the oxidation of several polyhydric phenols including catechol, homocatechol and pyrogallol, but was not active toward p-cresol, and catalyzed the oxidation of epinephrine only slightly.
Phenolases from plants and lower animals appear to be less specific in their actions than tyrosinase from mammals. Some plant enzymes catalyze the oxidation of a large number of different mono- and \( \alpha \)-dihydric phenols, and Sizer (21) presented evidence to support the contention that plant phenol oxidases act to catalyze the oxidation of tyrosine present in the peptide chain of certain proteins. Mammalian tyrosinase is more active in the presence of tyrosine and dopa than in the presence of other substances related structurally to these amino acids (8) although it has not been definitely determined if these substances are oxidized when attached in peptide chains. Natural substrates identified with phenolase include tyrosine (22), dopa (23), the tannins (24 & 25) and chlorogenic acid (26).

The oxidation of tyrosine and dopa by tyrosinase has been very widely investigated by Raper (27) and by Mason (28-30). The reactions involved in the enzymatic oxidation of dopa to melanin were summarized by Mason (29) and are shown in Figure 1. Mason (28-29) verified the presence of hallochrome (presently referred to as dopachrome) and Mason and Wright (30) showed that the rearrangement of this substance was pH dependent.

The most distinguishing character of phenol-oxidizing enzymes is their requirement for copper. All enzymes which have been sufficiently purified have proven to be copper-proteins and as such are inhibited by substances that bind copper. Kubowitz (31)
REACTIONS INVOLVED IN THE ENZYMATIC OXIDATION OF DOPA TO MELANIN (FROM MASON AND WRIGHT (30))

3,4-dihydroxyphenylalanine

enzyme

\[ \text{enzyme} \]

3,4-dihydroxyphenylalanine

\[ \rightarrow \]

dopa quinone

\[ \text{dopa quinone} \]

leuco dopachrome

\[ \text{leuco dopachrome} \]

\[ \text{dopachrome} \]

5,6-dihydroxyindole

\[ \text{5,6-dihydroxyindole} \]

\[ + \text{CO}_2 \]

\[ \rightarrow \text{OH}^- \]

5,6-dihydroxyindole-2-carboxylic acid

\[ \text{5,6-dihydroxyindole-2-carboxylic acid} \]

MELANIN

\[ \text{MELANIN} \]

FIGURE 1
first showed a linear relationship between the copper content of potato phenolase and its catecholase activity. He further demonstrated that copper-free protein no longer possessed enzyme activity by treating his preparations with cyanide and removing the copper-cyanide complex by dialysis. Other divalent metals such as iron, cobalt, nickel, manganese and zinc were found to be ineffective in restoring the enzyme activity when added to the copper-free protein.

Most inhibition studies carried out with phenol-oxidizing enzymes have involved substances which form weakly dissociable complexes with copper. These include sodium cyanide, hydrogen sulfide, carbon monoxide, diethyl-dithiocarbamate, sodium azide, \( p \)-amino-benzoic acid, and various sulfhydryl compounds (32). Other types of inhibitors are: (a) competitive inhibitors, including N-acetyltyrosine and other substituted tyrosine derivatives, (b) substances that combine with \( \sigma \)-dihydroxy groups such as sodium molybdate and triethanolamine titanate, (c) substances that combine with orthoquinones such as aniline and \( p \)-phenylenediamine, (d) reducing substances such as ascorbic acid and sulfites, and (e) hydroquinones such as \( p \)-hydroxy hydroquinone and \( p \)-benzylhydroquinone (32).

In certain instances, inhibitors have been used to retard activity during purification. Titanium complexes of lactic acid and triethanolamine have been used successfully during the purification of potato phenol oxidase (33).
Relatively small quantities of phenol oxidases have been found in all sources examined and consequently their complete purification has never been attained. From 5 to 10 mg. of the enzyme per pound of wet material (8) has been found in mushrooms _P. campestris_ and this is usually considered the best source for preparing the enzyme. This enzyme and those from other plants appear to be water-dispersible colloids and are easier to concentrate than those from mammalian sources. The enzyme(s) from the latter source are retained on ultramicroscopic cytoplasmic particles and as yet no method has been found by which the active enzyme can be separated from these particles (7).

The classical methods for protein separations have been used to purify phenol oxidases from many sources. These include cell rupture, organic solvent fractionation, freezing and thawing, salt fractionation and adsorption technics. The following investigators have purified phenol oxidases using various technics: Kubowitz (31 & 34) from white potato; Keilin and Mann (35), Ludwig and Nelson (36), and Mallette et al. (37) from the common mushroom _P. campestris_; Dalton and Nelson (38) from wild mushrooms _Lacatrius piperatus_; Eiger and Dawson (39) from sweet potatoes and Hogeboom and Adams (15) from mouse melanoma tumor.

There is always a loss in total activity during purification of these enzymes by methods outlined by the above investigators and in most instances the ratio of catecholase activity to cresolase activity increases during purification indicating that the monophenolase activity of
these preparations may be less stable than the o-dihydric phenolase activity (9 & 37).

Another important aspect concerning the stability of phenol oxidases is their apparent inactivation during catalysis. This inactivation is pronounced for preparations with high catecholase activity (38, 40 & 41). Most investigators believe that reaction products are not responsible for this inactivation since inactivation is still prevalent in the presence of ascorbic acid and other reducing agents which reduce o-quinones back to phenolic compounds (40). After studying activity kinetics, Asimov and Dawson (41) concluded that inactivation in the presence of pyrocatechol is not due to surface phenomena or reaction products.

Phenol oxidase activity may also be reduced or destroyed by elevated temperatures; however, activity may be enhanced with slightly elevated temperatures for phenolases from certain sources. Extensive studies by Bodine and co-workers (42-48) have established the interesting fact that phenol oxidases exist in certain invertebrates as an inactive precursor or proenzyme. This appears to be the only type of oxidizing enzyme occurring in the zymogen form. The "protyrosinase" isolated from the diapause eggs of the grasshopper (Melanoplus differentialis), larvae of the mealworm (Tenebrio molitor) and certain other invertebrates can be converted to the active form by a large variety of treatments, which have the common property of inducing protein denaturation.
CHAPTER III

QUANTITATIVE METHODS OF ANALYSIS

A. Activity Measurements

Two principal methods have been used in past investigations for determining tyrosinase activity. One involves determination by chemical means of either the rate of disappearance of the substrate or the rate of formation of some reaction product. The other measures by manometric technics the rate of oxygen uptake by the enzyme-substrate system.

Both colorimetric and manometric methods were used in the present investigation to study activities of shrimp phenolase(s). The actual method used depended to a large extent upon the type of substrate activity measured, but in general it was found that colorimetric methods were more practical. The methods used were as follows:

1. Catecholase Activity

A colorimetric method was used for comparing the catecholase activities of different fractions from shrimp during purification studies. This method seemed to offer the greatest speed, simplicity and sensitivity. It was based on the method of Smith and Stotz (49), for determining phenolase activity in plant material and depends on the reduction of either 2,6-dichlorobenzeneone-indo-3-chlorophenol or
2, 6-dichlorobenzene indophenol with hydrogen and palladium catalyst and oxidation of the leuco dye in the presence of enzyme and catechol.

The oxidized phenolic substrate is continually reduced by the leuco dye as follows:

\[
\begin{align*}
\text{(a)} & \quad \text{catechol} + \text{oxidase} \rightarrow \text{quinone} + \text{H}_2\text{O} \\
\text{(b)} & \quad \text{quinone} + \text{enzymatic reaction} \rightarrow \text{catechol} + \text{oxidized dye}
\end{align*}
\]

Reaction (b) is stoichiometric and rapid compared with (a) so that the overall reaction is limited by the enzymatic step. The reaction is followed by measuring the oxidized dye photometrically at 660 m\(\mu\) under conditions in which the quinone does not accumulate.

The exact procedure for determining catecholase activity by this method was as follows: five ml. aliquots of 0.0005 M leuco dye and 0.1 M phosphate buffer (pH 6.0) were transferred to Evelyn tubes, mixed and immersed in a constant temperature bath maintained at 35\(^\circ\)C \(\pm\) 0.1\(^\circ\)C. This mixture was shaken several times during the next 5 minutes and 1 ml. of enzyme added followed by the addition of 1 ml. of 0.1 M catechol (35\(^\circ\)C). The tube was quickly swirled for mixing and inserted in an Evelyn photoelectric colorimeter (660 m\(\mu\) filter) and the lamp rheostat adjusted to 100% transmittancy. Simultaneously, a stop watch was started, and
the tube immediately returned to the 35°C water bath. The mixture was shaken after 30 seconds to replenish oxygen and the galvanic reading recorded after 60 seconds. Similar procedures were followed to measure autoxidation of catechol and endogenous oxidation of the enzyme. For these determinations, the enzyme and catechol respectively were replaced by 1 ml. of water.

Relative catecholase activity was computed by converting percentage transmittancy after 1 minute to absorbance, subtracting the sum of the autoxidation of substrate and endogenous oxidation of enzyme from total oxidation and multiplying by $10^3$:

$$\text{Relative Activity} = \frac{(\Delta D_t) - (\Delta D_e + \Delta D_a)}{\Delta T} \times 10^3$$

Where:

- $\Delta D_t$ = Change in absorbance as measure of total oxidation,
- $\Delta D_e$ = Change in absorbance as measure of endogenous oxidation,
- $\Delta D_a$ = Change in absorbance as measure of autoxidation of substrate, and
- $\Delta T$ = Reaction time (1 minute).

Absolute or specific activity for catecholase as measured by this method was based either on quantity of non-dializable solids (mg./ml.) or on the amount of protein (mg./ml.). It was computed by dividing these quantities into the relative activity.
The pH had to be carefully controlled during activity measurements since a slight change in pH resulted in a large variation in activity. The effect of pH on catecholase activity under the conditions of this method is shown in Figure 2, where activity is plotted against pH. The enzyme preparation used in this experiment was obtained by blending _P. setiferus_ heads with an equal weight of cold (4°C) distilled water, freezing (-40°C) and thawing several times and centrifuging (2,600 X g., 10 min.). Curve (T) is the change in total activity of the supernatant solution as measured by the indophenol method with change in pH and demonstrates the continual rise in activity as the pH increased from 5.0 to 8.5. Curve (T-E) is similar to curve (T) except that the endogenous oxidation of the enzyme was subtracted from total oxidation. Curve (T-A) was obtained by subtracting autoxidation of the substrate from total oxidation, and curve T- (E+A) is the change with pH when the sum of autoxidation of substrate and endogenous oxidation of enzyme was subtracted from total oxidation.

These results show that the optimal pH for activity for this type of measurement is 6.8, but at this pH there was considerable autoxidation of substrate as well as endogenous oxidation of the enzyme. At pH 6.0 these oxidations were less pronounced and activity measurements were consequently more reproducible. The optimal temperature for measuring activity of this enzyme preparation at pH 6.0 was 35°C. (See Figure 3).
EFFECT OF pH ON ACTIVITY OF CATECHOLASE FROM CRUDE SHRIMP HEAD EXTRACT

T - Change in total activity with change in pH
T-E - Change in total activity less endogenous oxidation with change in pH
T-A - Change in total activity less autoxidation with change in pH
T=(E+A) - Change in exogenous oxidation with change in pH

FIGURE 2
CHANGE IN RELATIVE CATECHOLASE ACTIVITY
WITH CHANGE IN TEMPERATURE

FIGURE 3
When this enzyme preparation was diluted 1:2 and 1:4 and reaction rates compared with undiluted enzyme, it was found that the most valid values were those taken after one minute reaction time. Maximum difference between reaction rates (corrected for dilution and endogenous and autoxidation) determined in this manner was usually 10% in terms of absorbance provided the undiluted enzyme did not catalyze the oxidation of the leuco dye more than 0.25 absorbance units per minute. Typical results from this type of experiment are shown in Figure 4. Enzyme samples analyzed for catecholase activity in purification studies were always diluted so that the maximum change in absorbance for one minute reaction interval was 0.22.

The major disadvantage of this procedure for determining activity is that various other redox systems, both organic and inorganic interfere with the reaction. Several inorganic ions, particularly Cu$^{++}$ oxidize the leuco dye slightly under the conditions of catecholase measurement. As a means of reducing extraneous oxidations to a minimum, interfering trace ions were removed by ion exchange resins; Amberlite IR 4B-OH and IR 120-H. Distilled water was percolated first through an anion exchange column (Amberlite IR 4B-OH) followed by a cation exchanger (Amberlite IR 120-H). This water was free of copper, iron, cobalt and nickel as determined by spot test analysis (50), (dithiooxamide, copper; $\alpha$ - $\alpha$ -dipyridyl, iron; $\alpha$-nitroso-$\beta$ -naphthol, cobalt; and dimethylglyoxime, nickel), but was slightly
CHANGE IN ABSORBANCE WITH TIME DURING CATECHOLASE
DETERMINATION OF THREE SHRIMP HEAD PRESS JUICE SAMPLES

FIGURE 4
acid, having a pH of approximately 5.0. This was no disadvantage since all enzyme samples were buffered.

Advantages of this method other than simplicity, rapidity and sensitivity were: (a) it limited the amount of substrate used, so that the concentration remained high enough to support maximal activity of the enzyme, and (b) it minimized the possibility that a significant part of the enzyme would be inactivated during the reaction period, and this was important since the reaction temperature was relatively high.

2. Dopa Oxidase Activity

Dopa oxidase activity was also determined by a colorimetric method. This method depended on the oxidation of dopa to a red compound, 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome). This substance is the first colored intermediate formed in the oxidation of dopa by phenolase. It undergoes a base-catalyzed (non-enzymatic) decarboxylation and rearrangement, and subsequently is polymerized to melanin (27). At pH 6.0 or lower, the red compound is relatively stable (51) and it was shown by Horowitz and Shen (52) that the maximum rate of dopachrome production is proportional to the enzyme concentration in *Neurospora crassa* extracts.

The procedure for determining dopa oxidase activity colorimetrically was as follows: Seven ml. of 0.2 M sodium phosphate buffer (pH 6.0) and 2 ml. of 0.5% (w/v) gelatin were added to Evelyn
tubes and warmed to 35°C. Two ml. of substrate containing 3.5 mg. 
dopa per ml. were added followed by one ml. enzyme solution and the 
samples maintained at 35°C. for three hours. At the end of the re-
action period, the absorbance was determined photometrically in an 
Evelyn colorimeter at 470 mµ and compared with a blank containing 
enzyme, buffer and water. No correction was necessary for autoxi-
dation of the substrate at pH 6.0.

When a partially purified (see appendix I) enzyme was diluted 
and analyzed for dopa oxidase activity by this method and corrections 
made for protein dilution, the agreement was usually within 7%.

Table I shows absorbance values measured at various time intervals 
on enzyme preparations of different dilutions from representative ex-
periments. The best agreement in both cases was after three hours 
reaction time.

This colorimetric method for dopa oxidase activity was more 
convenient and considerably more sensitive than the manometric 
method, and consequently had greater applicability in measuring activities of dilute crude extracts, since the effects of interfering sub-
stances were minimized by using dilute preparations of the enzyme.

Relative dopa oxidase activity was computed by converting 
percentage transmittancy obtained after three hours to absorbance, 
subtracting endogenous oxidation of enzyme and multiplying the result 
by 10³.
TABLE I

COLORIMETRIC DETERMINATION OF DOPA OXIDASE ACTIVITY
OF DIFFERENT DILUTIONS OF ENZYME AT VARIOUS REACTION
INTERVALS

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Variation of absorbance after dilution correction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1 (No dil.) (1:1 dil.) (1:3 dil.)</td>
</tr>
<tr>
<td></td>
<td>Experiment 2 (No dil.) (1:1 dil.) (1:3 dil.)</td>
</tr>
<tr>
<td>10</td>
<td>0.1079 0.1062 0.1448 (1.6%)* 0.1412 0.2020 (3.1%)</td>
</tr>
<tr>
<td>20</td>
<td>0.1369 0.1412 0.2020 (34%)* 0.2020 (43%)</td>
</tr>
<tr>
<td>30</td>
<td>0.1549 0.1696 0.2220 (9.5%) 0.2220 (22%)</td>
</tr>
<tr>
<td>60</td>
<td>0.2147 0.2048 0.2620 0.1739 (4.6%) 0.1310 (23%)</td>
</tr>
<tr>
<td>90</td>
<td>0.2676 0.2556 0.2924 (4.5%) 0.2924 (6.3%)</td>
</tr>
<tr>
<td>120</td>
<td>0.3190 0.3036 0.3392 0.2596 (4.7%) 0.2616 (5.5%)</td>
</tr>
<tr>
<td>150</td>
<td>0.3570 0.3446 0.3768 (2.5%) 0.3768 (5.5%)</td>
</tr>
<tr>
<td>180</td>
<td>0.3980 0.3878 0.4204 0.328 (2.5%) 0.310 (5.5%)</td>
</tr>
</tbody>
</table>

*Per cent deviation from undiluted sample.
Relative Activity = \((\Delta D_t - \Delta D_e) \times 10^3\)

\(\Delta D_t\) = absorbance change during three hour reaction period in the presence of enzyme and DL-dopa.

\(\Delta D_e\) = absorbance change during three hour reaction period in the presence of enzyme without added substrate.

Specific activity for dopa oxidase was obtained by dividing the relative activity either by quantity of non-dialyzable solids (mg./ml.) or amount of protein (see later) in 1 ml. of enzyme sample.

3. Manometric Measurements of Shrimp Phenolase Activity

In our hands, manometric methods have been unsatisfactory for routine use in determining phenolase activities of shrimp samples during purification studies. Other investigators have also found that these methods are unreliable in purification studies (53) and some (35, 54-58) have resorted to other methods for determining activities of phenolases from various sources.

Manometric determinations of phenolase activity of shrimp samples is complicated by the release of carbon dioxide and ammonia. Carbon dioxide is apparently released by the action of decarboxylases in shrimp samples and was detected qualitatively by using Conway cells (59). Barium hydroxide was added to the outer wall of Conway cells and aqueous _P. setiferus_ head extract and DL-dopa added to the center well. After 1 hour, a considerable quantity of barium carbonate had formed in the outer wells of cells containing this reaction mixture but
the barium hydroxide in a control system which excluded the enzyme was only slightly turbid. Ammonia evolution was also demonstrated in a similar experiment by using Nessler's reagent (60) in the outer wells of Conway cells. Ammonia is believed to be released by the action of quinones on free amino acids or free amine groups of protein (61). The presence of ammonia and amines made pH control difficult and unless high buffer concentrations were used, the pH increased to 8.0 or more after a short reaction time.

In instances where manometric methods were used, oxygen uptake was measured in standard Warburg manometers using flat bottom 20 ml. reaction flasks. The temperature in most measurements was 25°C., the solutions were buffered with 0.4 M sodium phosphate buffer at pH 6.8, 0.1 ml. of 10% potassium hydroxide was used in the center well with the usual filter paper wick to increase surface area, and 1% gelatin used to retard inactivation of the enzyme during the reaction (62). One ml. of solution containing the required amount of substrate was added to the reaction flask followed by the addition of 1.9 ml. of buffer (pH 6.8) and 0.5 ml. of gelatin solution. Enzyme (0.5 ml.) was pipetted into a side arm, the manometers and flasks connected to the water bath and allowed to oscillate 70 oscillations per minute for 5 minutes. The system was then closed, the enzyme mixed with substrate and manometer readings taken at zero time. Subsequent readings were taken at 5 minute intervals for 20
minutes, then at 10 minute intervals for 1 hour or more depending on the activity of the enzyme.

In most instances, it was found that the enzyme preparations themselves absorbed oxygen in the absence of added substrate. It was necessary therefore to subtract this endogenous oxidation from total oxidation to obtain exogenous substrate oxidation. When oxygen uptake was measured in systems buffered at pH 6.8 or lower there was slight autooxidation of the various substrates used and this also was subtracted from total oxidation. The exogenous oxidation of the added substrate was obtained by subtracting the sum of the endogenous oxidation and the autooxidation of the substrate from total oxidation.

This method was used primarily to determine the substrate specificities, the effect of different substrate levels, and the effect of temperature changes on shrimp phenolase preparations.

B. Protein Determination

Several methods were used for protein determination; the method of choice depended largely on the quantity of sample available for analysis. When fractionations were made with large quantities of shrimp press juice or extracts, the dry weight of the non-dialyzable fraction was determined as follows: salt-free aliquots of each preparation were obtained by dialysis (see Appendix II) using several changes of copper-free water. The dialyzed sample was then quantitatively transferred to volumetric flasks. Aliquots, depending on
the quantity of protein, were then transferred to tared aluminum pans and dried to constant weight at 95°C.

Either the biuret method of Hiller, McIntosh and Van Slyke (63) for determining protein in urine or the sulfosalicylic acid method of Exton (64) was used for determining protein when the quantity of enzyme sample was limited. The sulfosalicylic acid method was used only when the sample volume was very small, such as in the various blood samples. Very good results were obtained with this method provided samples were diluted 1 to 150 and the sulfosalicylic acid then added to this diluted sample. Ovalbumin was used as a standard for both of these methods and the protein values determined by the sulfosalicylic acid method converted to equivalent quantities of biuret protein (i.e. protein as determined by biuret method) so that a more direct comparison could be made in determining specific activities. A conversion factor of 0.6 (Appendix III) was used to convert sulfosalicylic acid protein to biuret protein.

Specific activities were determined either on the basis of non-dialyzable solids or on the basis of biuret protein. One mg. of protein obtained by the biuret method was equivalent to 3 mg. of dry matter (see Appendix IV). Abbreviations are used throughout the remainder of this thesis to indicate specific activities of various enzyme fractions. These are as follows:
$C_w$ and $D_w$ represent catecholase and DL-dopa oxidase specific activities based on dry weights (mg. of non-dialyzable solid per ml. of sample).

$C_b$ and $D_b$ represent catecholase and DL-dopa oxidase specific activities based on quantity of protein (mg./ml.) determined by the biuret method (or converted to equivalent quantities of protein when the sulfosalicylic acid method was used)

C. Estimation of Copper

Samples used for copper analysis were dialyzed against frequently changed copper-free water for at least 72 hours to assure complete removal of non-bound copper. Enough copper-containing sample (usually 3 to 5 ml.) was added to a micro Kjeldahl flask to give 0.1 mg. to 0.3 mg. of copper. Two ml. of concentrated sulfuric acid and 5 ml. of concentrated nitric acid were added. Glass beads were used to prevent bumping and the samples were digested over a microburner. Heating was continued until solution volume was approximately 2 ml. and then extra nitric acid added as needed, followed by heating to digest completely the protein. The samples were carefully neutralized with 12 N sodium hydroxide, filtered and diluted to 10 ml. Copper was computed as percent in dry matter which was determined gravimetrically after heating for 12 hours at 95°C.

The colorimetric method of West and Compere (65) was used to determine copper in the digested sample. Dithio-oxamide was the
color reagent and interferences were removed with malonic acid. Gum arabic was used as a stabilizing agent.

Reagents:

A. Dithio-oxamide - dissolve 1 g. dithio-oxamide in 100 ml. 95% ethyl alcohol with heat.

B. Malonic Acid Conditioner - dissolve 100 g. malonic acid in 250 ml. distilled water, then add 14.0 g. sodium hydroxide and dilute to 500 ml.

C. Gum Arabic - A 1.0% aqueous solution of gum arabic was prepared daily.

To 10 ml. of digested sample 0.4 ml. malonic acid, 1.0 ml. gum arabic and 1.0 ml. dithio-oxamide were added and thoroughly mixed. After 15 minutes the samples were centrifuged to remove turbidity and absorbances determined in a Beckman DU spectrophotometer at 620 μν using a blank carried through the digestion process along with the sample. The quantity of copper was then estimated from a standard curve obtained under conditions similar to those used to determine absorbances of samples.
CHAPTER IV

EXPERIMENTAL AND DISCUSSION OF RESULTS

The demonstration that black spot in shrimp is caused by enzyme catalysis requires the identification and characterization of the enzyme system and a fundamental property of enzyme systems is that their reaction rates depend on the physical and chemical characteristics of the environment. Therefore, certain physical and chemical properties of shrimp tissue catecholase* were studied. These were: the role of copper in phenolase activity, inhibition of the enzyme, effects of heat and other stability factors on the activity of the enzyme, its energy of activation and Michaelis constants. The energy of activation and Michaelis constant for dopa oxidase activities of certain shrimp enzyme preparations were also studied.

Another factor which serves to identify certain enzyme systems is substrate specificity. The catalytic effects of different shrimp tissue preparations on the mono- and dihydric phenols

*Pyrocatechol (catechol) was used as substrate during most of these studies since previous workers have used this substance when studying phenolase activities quantitatively, and certain properties of catecholases are known.
usually associated with phenolase activity were determined. Natural substrates in shrimp were also studied in an attempt to clarify the nature of reactions leading to black spot formation.

The anatomical distribution of shrimp phenolase was studied to localize the most highly active tissues which could be used in purification of enzymes causing melanosis.

A. Role of Copper in Shrimp Catecholase Activity

All enzymes thus far identified as phenolases by various investigators required copper for their activities. This cofactor is specific (31) and activity is lost when copper is replaced by other metal ions.

By an experiment similar to those of Kubowitz (31), it was found that the removal of copper brought about a decrease in catecholase activity of shrimp protein and that addition of copper reactivated the enzyme. An enzyme sample from *P. setiferus* press juice (Appendix V) was dialyzed for 24 hours against copper-free water and a small portion was frozen. The remainder of the solution was dialyzed against 0.1 M sodium cyanide followed by 24 dialysis against copper-free water. The dialyzate was divided into three equal portions and frozen for several days. All four samples were then thawed and to one of the cyanide treated samples 0.5 ml. of copper sulfate containing 250 μg.Cu^{++} was added; 0.5 ml. of solution containing 10 μg.Cu^{++} was added to a second portion of cyanide treated sample, and 0.5 ml. of copper-free water added to the third portion.
Catecholase activity was then determined manometrically at pH 6.8 and 25°C. in the presence of $2 \times 10^{-1}$ M pyrocatechol as outlined in Chapter III. The quantity of oxygen absorbed by each sample was as follows:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oxygen Absorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-treated</td>
<td>5.2 µl. O$_2$/min.</td>
</tr>
<tr>
<td>CN$^-$ treated + 250 g. copper</td>
<td>3.7 µl. O$_2$/min.</td>
</tr>
<tr>
<td>CN$^-$ treated + 10 g. copper</td>
<td>3.2 µl. O$_2$/min.</td>
</tr>
<tr>
<td>CN$^-$ treated + water</td>
<td>0.9 µl. O$_2$/min.</td>
</tr>
</tbody>
</table>

Removal of copper by dialyzis against cyanide and water resulted in approximately 85% loss in activity of catecholase, but the addition of copper stimulated activity to about 60% of the original activity. Excess copper at this pH did not greatly affect activity.

These results demonstrate the necessity of copper in shrimp catecholase activity and this is further substantiated in the following section which reports results of catecholase inhibition by various substances including copper-sequestering agents.

B. Inhibition of Shrimp Catecholase and Dopa Oxidase

Reducing agents are the most practical inhibitors of melanin formation in shrimp (66 & 67), and sodium bisulfite is presently being used commercially to retard black spot formation, but it is difficult to show this inhibition quantitatively. Sodium bisulfite and other reducing agents interfere with the indophenol method for determining catecholase activity and the effect of most reducing agents cannot be
measured manometrically since they either evolve gases or absorb oxygen in the presence of shrimp extracts and are oxidized. However, it was shown by using the colorimetric method described in Chapter III that 0.01 M ascorbic acid inhibited dopa oxidase activity in _P. azte cus_ press juice (Appendix V) almost completely (95%). This enzyme activity was also inhibited 86% in the presence of 0.005 M sodium bisulfite. The inhibition of catecholase activity (indophenol method, Chapter III) of this juice by other types of chemicals was studied quantitatively on _P. azte cus_ head press juice (Appendix V) and the results given in Table II.

Substances that bind copper were effective inhibitors of catecholase activity and this is further evidence for the necessity of copper in catecholase activity. The inhibition of endogenous oxidation by these substances indicates that at least part of the endogenous oxidation is caused by the catalytic oxidation of natural substrates by copper-containing or copper-requiring enzymes.

Carboxy acids were shown by Krueger (68) to inhibit phenolase activity at low pH values and this was attributed to complexation of some active center of the enzyme by the carboxylic acid anion. Triethanolamine titanate complexes dihydric phenols and thus prevents their oxidation by the enzyme.

Cysteine (0.01 M) completely inhibited dopa oxidase activity of _P. azte cus_ head press juice and this can possibly be explained
## TABLE II

**CHEMICAL INHIBITION OF CATECHOLASE ACTIVITY AND ENDOGENOUS OXIDATION OF SHRIMP HEAD PRESS JUICE**

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH²</th>
<th>Method</th>
<th>Percent (Total)³</th>
<th>Inhibition (Endogenous)²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substances which combine with copper</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diethyldithiocarbamate (0.017 M)</td>
<td>6.0</td>
<td>Manometric⁴</td>
<td>36</td>
<td>70</td>
</tr>
<tr>
<td>Thiourea (0.02 M)</td>
<td>5.9</td>
<td>&quot;</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Cysteine (0.012 M)</td>
<td>6.0</td>
<td>&quot;</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td><strong>Carboxy acids or their salts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium EDTA³ (0.0002 M)</td>
<td>6.5</td>
<td>Indophenol</td>
<td>45</td>
<td>50</td>
</tr>
<tr>
<td>Sodium benzoate (0.0002 M)</td>
<td>6.6</td>
<td>&quot;</td>
<td>46</td>
<td>23</td>
</tr>
<tr>
<td>Calcium acetate (0.012 M)</td>
<td>6.0</td>
<td>&quot;</td>
<td>7</td>
<td>--</td>
</tr>
<tr>
<td>Oxalic acid (0.002M)</td>
<td>5.6</td>
<td>Manometric</td>
<td>97</td>
<td>27</td>
</tr>
<tr>
<td><strong>Substance that complexes phenols</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAT-21 (0.002M)</td>
<td>6.0</td>
<td>Manometric</td>
<td>46</td>
<td>1</td>
</tr>
</tbody>
</table>

a - pH after reaction  
⁰ - (Ethylendinitrilo) tetra acetic acid - (sodium salt)  
C - Triethanolamine titanate  
⁴ - Manometric determinations were made as outlined in Chapter III in the presence of 4 X 10⁻² M pyrocatechol.  
e - Inhibition of total oxidation in presence of added pyrocatechol.  
f - Inhibition of endogenous oxidation (no pyrocatechol added).
on the basis of its ability to sequester copper. Cupric ions react with cysteine and other sulfhydryl group containing compounds both in acid and alkaline solutions to form yellow or yellow-brown insoluble copper sulfides(69).

\[ \text{Cu}^{++} + 2R-S^{-} \rightarrow \text{Cu}(RS)_2 \]

Cysteine forms a brownish-black precipitate with cupric ions at pH 6.0 and undoubtedly acts primarily as a copper sequestering agent in the inhibition studies mentioned above. Nevertheless, the possible role of cysteine as a reducing agent should not be overlooked in inhibition of phenolase activity.

Reducing substances such as the sulfites, ascorbic acid and cysteine are useful in the control of black spots in shrimp because they immediately reduce o-quinones and retard melanin formation. As long as adequate reducing groups are available in shrimp, quinones are not formed and melanosis is prevented.

Sulfhydryl and other reducing groups and reducing enzymes present in shrimp may serve to maintain the redox potential sufficiently low in live shrimp to prevent melanosis. This possibly explains why shrimp shells do not blacken in live shrimp although both substrate and phenolase are apparently present.

C. Stability and Inactivation of Shrimp Catecholase

Another type of study which is of considerable importance in the characterization of enzymes is the rate of thermal inactivation.
The velocity constants and half-lives at various temperatures are measures of the degree of thermal stability of an enzyme.

The thermal stability of shrimp catecholase was determined at 35°C. for periods of time from 30 minutes to 7 hours. Three grams of lyophilized powder from *P. setiferus* heads described in Appendix I (sample B_p) were ground to a paste with 5 ml. distilled water and dispersed in 25 ml. of distilled water. This was centrifuged (2,600 x g, 10 min.) and a portion of the supernatant incubated with 0.2 M sodium phosphate buffer (pH 6.0) in the proportion of 1 part enzyme to 5 parts buffer (maintained at 35°C. prior to mixing). Two 6 ml. aliquots were removed after 30 minutes and after 1 hour and then hourly for 7 hours. The samples were immediately cooled in ice water and kept cool until analyzed for catecholase activity by the indophenol method. This was done by warming the samples individually for 45 seconds at 35°C., adding 5 ml. aliquots of leuco indophenol dye (previously warmed to 35°C.), and finally adding 1 ml. of 0.1 M pyrocatechol. The usual procedure for determining catecholase (Chapter III) was then followed, and endogenous oxidation subtracted from total oxidation to obtain relative catecholase activity.

The straight line obtained by plotting the logarithm of relative catecholase activity against heating time at 35°C. is shown in Figure 5. The inactivation of catecholase activity between 30 minutes and 6 hours was approximately first order since the relationship of
STABILITY OF SHRIMP CATECHOLASE AT 35° C.

Figure 5

Heating Time at 35° C. (Hours)

Relative Activity x 10^-3
time vs. activity decreased linearly during this period. The enzyme was inactivated almost completely after seven hours under these conditions and its half-life was approximately 1 hour.

A further experiment was conducted on crude press juice from *P. aztecs* heads to determine the effects of heating at various temperatures on catecholase activity. A crude enzyme solution was prepared by pressing the juice from the heart and stomach areas of shrimp heads. The juice was frozen, thawed, and centrifuged (2,600 X g, 20 min.) and the supernatant decanted. The top layer of the insoluble material contained numerous leucocytes and this was suspended in the supernatant. The relative catecholase activity of this sample was 211. Three ml. aliquots were added to Evelyn tubes containing 15 ml. of 0.2 M sodium phosphate buffer (pH 6.0). Samples were heated to 40, 50, 60, 70, 75 and 80°C as follows: the tubes were immersed individually in a water bath and the solution gently stirred with a thermometer. After the appropriate temperature was reached the tubes were maintained at the temperature for one minute. They were then immersed in an ice bath and stirred to hasten temperature lowering. Samples were then examined individually at 35°C for catecholase activity by the indophenol method.

Another similar experiment was conducted to determine the degree of thermal inactivation of a relatively pure sample of phenolase taken from *P. setiferus* hearts. This sample was prepared by
grinding shrimp hearts with sand in 0.1 M phosphate buffer at pH 6.0, freezing and thawing, centrifuging and dialyzing. The sample was then diluted 1 to 5 with cold copper-free water, filtered through a 0.5 cm. thick pad of celite (L-665 filter aid) and finally centrifuged (4,500 X g., 10 minutes). There were no leucocytes in the supernatant solution and its relative catecholase activity was 182.

Figure 6 represents the effect of heat on the catecholase activities of these two preparations. Lines 1 and 2 are drawn to indicate the relative activities of the two preparations prior to heating (i.e. 211 and 182 respectively). Curve C depicts the effect of heat on the crude preparation and curve P the effect of heat on the purified preparation. It can be noted from these curves that heating the crude preparation for 1 minute between 40 and 60 degrees centigrade resulted in increased catecholase activity. The maximum increase over the non-heated sample was 12.3% and was obtained by heating 1 minute at 50°C. Heating the purified sample 1 minute at 50°C. also resulted in increased catecholase activity (4.4%). A further experiment in which the purified sample was heated 2 minutes at 50°C. resulted in a 26% increase in catecholase activity.

These findings indicate that the catecholase in these preparations was heat-activated at certain temperatures. It is possible that some of this increase in activity of the crude sample was caused by rupture of leucocytes which released enzyme, but this
EFFECT OF TEMPERATURE ON CATECHOLASE ACTIVITY OF SHRIMP HEAD PRESS JUICE AND PURIFIED HEART EXTRACT

1 - Activity of *P. aztecus* head press juice before heating
2 - Activity of *P. setiferus* heart extract before heating
C - Effect of heating on *P. aztecus* head press juice catecholase activity
P - Effect of heating on *P. setiferus* heart extract catecholase activity

FIGURE 6
does not explain the increase in catecholase activity of the blood sample which contained no leucocytes. In view of the extensive investigations of Bodine and co-workers (42-48) it is possible that part of the catecholase in the shrimp samples could be proenzymatic in nature and active centers exposed by heating. Results from preliminary experiments concerning the effects of different inorganic salts on catecholase activity also supported the idea of the existence of prophenolase in shrimp extracts and blood.

The stability of shrimp catecholase was characterized further during purification studies of the enzyme. Catecholase activity was not diminished by cold fractionation with ammonium sulfate or by extracting with sodium chloride, sodium phosphate or sodium carbonate. Crude press juice catecholase from shrimp heads (Appendix V) was stable to lyophilization and in some instances its activity increased after this treatment. Catecholase activity was diminished when the enzyme was precipitated with cold acetone or alcohol, or with acid at its isoelectric point (pH 4.8).

Freezing at -13°C. and thawing decreased activities of samples that had been partially purified by ammonium sulfate fractionation and dialysis, but if crude extracts or press juice from heads were stored at freezing temperatures and thawed, the activity usually increased. Subsequent freezing and thawing of such samples often resulted in further increased activity. The enzyme was quite
stable at 4°C. if its solutions were saturated with toluene prior to storage, but slowly lost activity if kept at room temperature under these conditions.

D. Energy of Activation

Another thermal effect concerned with enzyme activities is the energy of activation or the rate of reaction increase for a fixed increase in temperature. In inorganic catalysis, the energy of activation of the reaction is characteristic of the catalyst and is independent of the substrates when related substances are used (70). The activation energy of enzyme systems then should be independent of different environmental changes unless these factors alter the catalytic surface of the enzyme in some manner.

For yeast invertase, the activation energy was found (71) to be essentially unaffected by (a) changes in pH from 3.2 to 7.9, (b) changes in electrolyte concentration, and (c) changes in the concentration of enzyme or substrate. Also, values for E are quite constant over a wide range of temperatures until inactivation is apparent (72). The purity of the enzyme plays no role in determining the activation energy, since identical E values were obtained for crude and partially purified aldehyde-purine dehydrogenase of milk (73) and for soybean urease in different stages of purification (74).
Since activation energy of enzymes is considered a constant which is independent of enzyme purity, it should be relatively constant for enzymes that catalyze the same reaction even though their sources may differ. Gould (75) substantiated this belief when he reported a value of 2,700 calories/mole as energy of activation for phenolase preparations from Irish potato, mushroom (P. campestris), and meal worm (Tenebrio molitor) acting either on catechol or on p- cresol. This suggests a close similarity of phenolases from various sources and that a single enzyme is involved in both the mono- and o-dihydric phenolase activity of these enzymes.

The activation energy was determined for a crude extract of shrimp phenolase by measuring the catecholase activity both manometrically and colorimetrically (indophenol method).

Press juice from the gill area of shrimp heads was frozen and thawed several times and then centrifuged (27,000 X g., 10 min.) to remove lipid, pigments and insoluble protein. Five 20 ml. portions were frozen and thawed as needed to determine the activities at various temperatures.

The manometric procedure for determining catecholase activity was similar to that of Gould (75) except that the sodium phosphate buffer concentration was 0.4 M. The final substrate concentration was 2 x 10^{-1}M and potassium hydroxide was used in the center well to remove carbon dioxide. The pH was 6.0 and the total volume 4.0 ml. Since standard Warburg manometers were used to measure oxygen uptake, the endogenous oxidation and autoxidation of substrate were subtracted from total oxidation to obtain exogenous substrate oxidation.
Figure 7 illustrates plots of oxygen uptake obtained for catecholase activity at various temperatures. The rate of oxygen consumption was computed from the slope of the straight line portions of these curves. The data between 10 and 30°C are used in the form of an Arrhenius plot (log. of activity rate* plotted against reciprocal of absolute temperature) (Figure 8). The energy of activation calculated from the slope of the straight line of this plot was 3,000 calories/mole.

An Arrhenius plot of data obtained for catecholase activity by the indophenol method is also shown in Figure 8. The energy of activation computed from the slope of this curve was 5,200 calories/mole. No exact agreement was expected between this value and that determined by the measurement of oxygen uptake. The manometrically determined energy of activation closely agrees with values obtained by Gould (75) for energies of activation of phenolases from a variety of sources and falls in a very low range as far as activation energies are concerned.

These values in general suggest a high activity for these enzymes and also support the belief (12) that phenolases are oxygen

*Total oxygen uptake minus sum of autoxidation and endogenous oxidation per minute.
CHANGE IN OXYGEN UPTAKE WITH TEMPERATURE FOR SHRIMP HEAD PRESS JUICE CATECHOLASE

FIGURE 7
ARRHENIUS PLOTS OF CATECHOLASE ACTIVITIES MEASURED
BY COLORIMETRIC AND MANOMETRIC METHODS

FIGURE 8
transferases (aerobic dehydrogenases) rather than anaerobic dehydrogenases. This is evidenced by the fact that most anaerobic dehydrogenases have activation energies ranging from 15,000 to 25,000 calories/mole (72).

E. Michaelis Constants

The activity of enzymes in converting substrate to products depends on the availability of substrate as well as the time required for the enzyme to react with substrate and release products. The more abundant the substrate molecules surrounding the enzyme, the shorter the time during which the enzyme or part of the enzyme is left uncombined and therefore not active. At some definite substrate concentration the enzyme is said to be saturated and activity proceeds at a maximum under conditions of the activity measurement.

The Michaelis constant is a measure of the substrate concentration required to give half the maximum velocity of an enzyme under definite conditions and is considered by many investigators as a constant for enzymes from different sources that catalyze the same reaction. Any factor that varies the velocity constant for combination of enzyme and substrate such as pH, ionic strength, temperature, etc., will also vary the Michaelis constant. Then, substrate level at half-maximum activity can be used to characterize an enzyme provided conditions are rigidly controlled and specified.
Instances in which Michaelis constants have been reported in the literature for phenolase activity are rare, but some have been given. Heyman et al. (76) working with potato phenolase determined constants for both catecholase and \( p \)-cresolase activities of the enzyme. Under the conditions of their experiment using the chronometric method of Miller et al. (54) they found the half-maximum velocity for catecholase at a substrate concentration of \( 4.4 \times 10^{-3} \) moles/liter, and using a manometric method a constant of \( 1.7 \times 10^{-3} \) moles/liter (computed from data reported in their paper) for \( p \)-cresolase activity. The only other constants for phenolases available in the literature are those reported by Horowitz and Fling (77) for oxidase activity of thermostable and thermolabile \( L \)-tyrosinase and \( L \)-dopa oxidase from Neurospora. These investigators determined activities manometrically and found the following values for \( K_m \):

- Thermostable enzyme, \( L \)-tyrosinase; \( 1.52 \times 10^{-3} \) moles/liter
- Thermolabile enzyme, \( L \)-tyrosinase; \( 1.92 \times 10^{-3} \) moles/liter
- Thermostable enzyme, \( L \)-dopa oxidase; \( 1.29 \times 10^{-3} \) moles/liter
- Thermolabile enzyme, \( L \)-dopa oxidase; \( 1.28 \times 10^{-3} \) moles/liter.

Three grams of lyophilized powder from \( P. \) setiferus shrimp described in Appendix I, sample B, were ground to a paste with 5 ml. distilled water and then dispersed in 25 ml. of distilled water. This was centrifuged (2,600 \( \times g \), 10 min.) and the supernatant used to determine Michaelis constants for catecholase and DL-dopa oxidase activities. The constant for catecholase activity was determined by
two methods: the indophenol colorimetric method and the manometric method. For the latter method, the pH was lowered to 6.0 so that conditions of the two methods would be more nearly equal.

DL-dopa oxidase activity was determined as described in Chapter III. The substrate concentrations were varied as shown in Table III and Michaelis constants determined graphically from plots of $1/v$ versus $1/(S)$ according to the method of Lineweaver and Burk (78). A value of $3.65 \times 10^{-2}$ moles/liter was found by the indophenol method for catecholase activity and $5.0 \times 10^{-2}$ moles/liter by the manometric method. The constant for DL-dopa oxidase activity was $3.78 \times 10^{-3}$ moles/liter.

No valid comparisons can be made between constants reported here and those given in the literature since the methods and conditions for measuring activities were not the same. The two constants for catecholase activity however are in good agreement considering the differences in the two methods used to determine activity.

F. Substrate studies

1. Phenolase Specificity of Various Shrimp Enzyme Preparations.

Experiments were carried out to determine the substrate specificities of phenolases in various shrimp enzyme preparations by measuring oxygen uptake manometrically in the presence of shrimp enzyme and various phenols.
### TABLE III

**DATA FOR DETERMINING MICHAELIS CONSTANTS FOR O-DIHYDRIC PHENOLASE ACTIVITIES OF CRUDE SHRIMP EXTRACTS.**

**Catecholase activity (Indophenol method)**

<table>
<thead>
<tr>
<th>(S) M</th>
<th>1/ (S)</th>
<th>v</th>
<th>1/v</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Relative Activity $\times 10^{-3}$</td>
<td></td>
</tr>
<tr>
<td>0.1000</td>
<td>10</td>
<td>1.172</td>
<td>0.853</td>
</tr>
<tr>
<td>0.0500</td>
<td>20</td>
<td>0.879</td>
<td>1.140</td>
</tr>
<tr>
<td>0.0250</td>
<td>40</td>
<td>0.649</td>
<td>1.542</td>
</tr>
<tr>
<td>0.0125</td>
<td>80</td>
<td>0.359</td>
<td>2.790</td>
</tr>
<tr>
<td>0.0050</td>
<td>200</td>
<td>0.218</td>
<td>4.580</td>
</tr>
</tbody>
</table>

**Catecholase activity (Manometric method)**

<table>
<thead>
<tr>
<th>(S) M</th>
<th>1/ (S)</th>
<th>v $\mu \text{O}_2/\text{Min.}$</th>
<th>1/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1000</td>
<td>10</td>
<td>2.00</td>
<td>0.50</td>
</tr>
<tr>
<td>0.0500</td>
<td>20</td>
<td>1.47</td>
<td>0.68</td>
</tr>
<tr>
<td>0.0250</td>
<td>40</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>0.0167</td>
<td>60</td>
<td>0.64</td>
<td>1.38</td>
</tr>
<tr>
<td>0.0125</td>
<td>80</td>
<td>0.50</td>
<td>2.00</td>
</tr>
</tbody>
</table>

**DL-Dopa oxidase activity (Colorimetric method)**

<table>
<thead>
<tr>
<th>(S) M</th>
<th>1/ (S)</th>
<th>v</th>
<th>1/v $\times 10^{3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Relative Activity</td>
<td></td>
</tr>
<tr>
<td>0.01000</td>
<td>100</td>
<td>561</td>
<td>1.78</td>
</tr>
<tr>
<td>0.00500</td>
<td>200</td>
<td>411</td>
<td>2.44</td>
</tr>
<tr>
<td>0.00250</td>
<td>400</td>
<td>312</td>
<td>3.22</td>
</tr>
<tr>
<td>0.00125</td>
<td>800</td>
<td>202</td>
<td>4.95</td>
</tr>
</tbody>
</table>
The sources of the enzyme preparations were abdomen shells from \textit{P. setiferus} and \textit{P. duoarum}, the antennae from \textit{P. aztecus} and blood from \textit{P. setiferus}. The abdomen shells were removed from several shrimp, rinsed in distilled water, blotted on Whatman no. 1 filter paper and cut into small pieces. Both species of shrimp had been refrigerated (ice) for over a week before analysis. The antennae used were frozen (-50°C.) and ground to a fine powder with mortar and pestle. The press juice was obtained by severing the gill area from freshly caught \textit{P. aztecus} heads, pressing the fluids from them, freezing and thawing several times and finally removing insoluble solids by centrifuging (2,600 X g., 10 min.). \textit{P. setiferus} blood was obtained by aspirating from the pericardial sinus area of live shrimp. It was stored at -3°C. until used.

The manometric procedure used in these studies was principally that outlined in Chapter III. Duplicate 0.25 g. samples of shell or antennae and 0.5 ml. samples of press juice or blood were added to Warburg flasks and buffered with 0.4 M sodium phosphate buffer (pH 6.8). Potassium hydroxide was used to absorb carbon dioxide. The substrates (in 1 ml. of copper-free water) were added from side arms after shaking in an open system at 25°C. for 5 minutes. The system was closed and manometric readings taken as outlined previously. Activity was based on total oxygen uptake during a three hour reaction period. The endogenous oxidation in
The absence of substrate was subtracted from the total oxidation (oxidation in presence of added substrate) and the reaction rate computed as microliters of oxygen taken up per gram of dry weight (dried 95\(^\circ\) C., 8 hours) per hour. Data concerning substrate concentrations, quantity of enzyme, source of enzyme and oxygen uptake for the enzyme preparations in the presence of the various substrates are presented in Table IV.

Enzymes in abdomen shell and adhering cuticle catalyzed oxygen uptake at a greater rate in the presence of \(o\)-dihydric phenols than in the presence of monophenols. The press juice from \(P.\) aztecus heads exhibited unusually high cresolase activity. It also catalyzed the oxidation of DL-tyrosine at a greater rate than it did an equal molar quantity of DL-dopa, indicating a high monophenolase activity. In no other instance was such a high cresolase activity encountered. \(P.\) setiferus blood and \(P.\) aztecus antennae also catalyzed the oxidation of both mono- and \(o\)-dihydric phenols.

The high rate of oxygen uptake of \(P.\) aztecus press juice and \(P.\) duoarum abdomen shells in the presence of hydroquinone indicates that a low concentration of \(o\)-benzoquinone may be necessary during activity of phenolases as suggested by Adams and Nelson (79). Hydroquinone is believed to act as a reducing agent to reduce \(o\)-quinones similar to the indophenol-\(o\)-quinone redox system used for measuring catecholase activity (Chapter III). Hydroquinones and the resorcinols
# TABLE IV

**OXYGEN UPTAKE FOR ENZYME PREPARATIONS IN THE PRESENCE OF VARIOUS SUBSTRATES**

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Shrimp Species</th>
<th>Substrate and Concentration</th>
<th>Oxygen Uptake $\mu l/g./hour$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdomen shells</td>
<td><em>P. setiferus</em></td>
<td>DL-dopa ($C_3$) 270</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pyrocatechol ($C_5$) 260</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-cresol ($C_6$) 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DL-tyrosine ($C_4$) 50</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td><em>P. setiferus</em></td>
<td>L-dopa ($C_1$) 330</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DL-dopa ($C_1$) 165</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-cresol ($C_1$) 330</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>hydroquinone ($C_1$) 125</td>
<td></td>
</tr>
<tr>
<td>Press juice</td>
<td><em>P. aztecus</em></td>
<td>L-dopa ($C_1$) 220</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DL-dopa ($C_1$) 260</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pyrocatechol ($C_1$) 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DL-tyrosine ($C_2$) 180</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-cresol ($C_1$) 720</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>hydroquinone ($C_1$) 300</td>
<td></td>
</tr>
<tr>
<td>Antennae</td>
<td><em>P. aztecus</em></td>
<td>DL-dopa ($C_3$) 480</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-cresol ($C_6$) 504</td>
<td></td>
</tr>
<tr>
<td>Abdomen shells</td>
<td><em>P. duoraum</em></td>
<td>DL-dopa ($C_3$) 350</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pyrocatechol ($C_5$) 304</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-cresol ($C_6$) 68</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>m-cresol ($C_6$) 54</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>hydroquinone ($C_2$) 408</td>
<td></td>
</tr>
</tbody>
</table>

$C_1 = 2.0 \times 10^{-3}$ M; $C_3 = 5.0 \times 10^{-3}$ M; $C_5 = 9.1 \times 10^{-3}$ M;

$C_2 = 3.4 \times 10^{-3}$ M; $C_4 = 5.5 \times 10^{-3}$ M; $C_6 = 9.3 \times 10^{-3}$ M.
are not considered substrates for phenolases but are oxidized in the presence of o-quinones.

L-dopa was oxidized approximately twice as fast as equal molar quantities of DL-dopa in the presence of blood from \textit{P. setiferus} and press juice from \textit{P. aztecs} heads. It is possible that the phenolase involved is stereospecific for the oxidation of the L-isomer of this substrate.

2. Natural Substrates for Shrimp Phenolase(s)

Evidence was obtained which indicates that phenolases in shrimp oxidize phenolic groups attached to proteins or polypeptides as well as free phenols. When shrimp extracts or blood fractions were dialyzed for several days to remove amino acids and other water soluble materials, most preparations continued to darken; and if held at 25\degree C. or above, darkening was appreciable after short intervals of time. These solutions first turned pink, then red, and finally black. Shell cuticle and blood proteins gave strong phenol tests with ferric chloride and with modified Folin and Ciocalteu reagent (80). The epicuticle attached to shell darkened considerably when held overnight at room temperature in the presence of shrimp blood taken from the pericardial sinus of \textit{P. aztecs} shrimp.

There is little doubt that some phenolases can catalyze the oxidation of phenols attached to protein (21), but it has not been
shown that these groups can be converted to melanin. Since no preparation examined was sterile, bacteria present might have been producing proteolytic enzymes which acted in conjunction with phenolase to produce non-dialyzable melanin.

In an effort to identify natural substrates involved in black spot formation, a paper chromatographic procedure similar to that used by Bailey (81) for the separation and identification of sugars was used to separate the amino acids in samples of press juice from heads and blood of *P. setiferus*. The organic fraction of a mixture of 1-butanol, acetic acid and water (50:10:40) was used as solvent in a chamber designed for ascending paper chromatography.

A 20 μl aliquot of the various samples was applied to Whatman no. 1 filter paper along with standard amino acids (50 μg.). The chromatograms were placed in solvent saturated chambers and the solvent allowed to ascend the paper for 12 hours. The air dried chromatograms were dipped in ninhydrin, dried at room temperature and heated at 85° C. for 5 minutes. The amino acids were identified by Rf values and by comparing their rates of movement with those of the standard acids.

L-tyrosine was invariably present in these samples and L-dopa was identified in several samples.

The method outlined by Clark et al. (82) for fractionating polyphenols in potatoes was used to extract and isolate polyphenols
from shrimp head press juice. This method involves extraction with acetone, concentration and precipitation of phenols as lead phenolates, removal of lead with sulfuric acid and extraction with ethyl acetate. The ethyl acetate solutions were then evaporated and the residue extracted with dilute (0.01%) acetic acid. Tests with modified Folin and Ciocalteu reagent (80) indicated the presence of phenols, but the ninhydrin test for amino acids (69), page 207-210 was negative. An absorption spectrum of the acidic solution exhibited absorption peaks at 272 m\(\mu\) and between 300 m\(\mu\) and 307 m\(\mu\). The only phenolic compound known to absorb in these regions is 5, 6-dihydroxy indole which was found by Mason (29) to absorb maximally at 275 m\(\mu\) and 298 m\(\mu\). No further attempt was made to identify this compound.

Pressed juice from *P. aztecs* heads was diluted 10 to 100 ml. with 0.4 M. sodium phosphate buffer and 10 ml. dopa (1 mg./ml. also in buffer) added. Oxygen was bubbled through this solution for two minutes and the flask stoppered. The solution turned red-brown after two hours at 25\(^{\circ}\) C. and absorbed strongly at 280 m\(\mu\) and 475 m\(\mu\). After five hours at this temperature the solution turned black.

This sequence of color changes is exactly that described by Mason (29) for the three chromophoric phases brought about by the action of meal worm tyrosinase on dopa. The material
absorbing at 475 m\(\mu\) was characterized by Mason (29) as hallochrome red (dopachrome). When diluted shrimp head press juice was held under similar conditions in the absence of added dopa the same sequence of color changes occurred, and the red-brown solution absorbed strongly at 475 m\(\mu\). Absorbance in this range indicates the presence of hallochrome red.

Since tyrosine was present in quantity, and dopa formed under natural conditions and appears to be converted to dopachrome, tyrosine apparently is a natural product oxidized in a sequence of reactions leading to black spot formation. The ease of dopachrome formation in the presence of exogenous dopa and the possible identification of 5,6-dihydroxy indole as an intermediate product is evidence that the tyrosine is converted to black spot and that these are actually "dopa melanins".

G. Purification of Shrimp Head Catecholase

Results reported in the previous section concerning the substrate specificity of shrimp phenolase indicate that the best sources of the enzyme(s) for purification purposes were the antennae, the abdomen shells and blood. Several attempts were made to purify catecholase from the abdomen shells and antennae but very little purification was attained for enzyme samples from these sources even though a variety of methods were used.
Press juice and extracts from heads were the most practical sources for crude enzyme and were used in attempts to isolate catecholase. The methods used in these studies were those commonly employed including lyophilization, salt fractionation, low temperature organic solvent precipitation and adsorption and elution procedures. The maximum increase in purity achieved through use of these methods was 17 fold represented by a specific activity of $C_p 130$. Since this value, as pointed out later, is lower than catecholase activities obtained from certain blood fractions from shrimp, blood is obviously a better initial source for fractionation studies and will undoubtedly be used in future attempts to purify this enzyme. Quantities of shrimp blood available during the progress of work reported in this dissertation were insufficient for thorough purification studies. Detailed descriptions of two of the many schemes used in attempts to purify catecholase from various head extracts are given in Appendix VI.

H. Phenolase in Shrimp Blood

While studying the substrate specificities of phenolase(s) from various anatomical locations of shrimp it was found that rinsed abdomen shell (and/or the firmly attached cuticle) including the telson and uropod had both mono- and $\alpha$-dihydric phenolase activities. It appeared that these tissues alone contained sufficient quantities of both enzyme and substrate to cause the melanosis
observed in refrigerated or frozen shrimp abdomen (tail), but it was also observed that the contents of the cephalothorax turned black very rapidly, even if kept at refrigerated temperatures and particularly if whole shrimp were frozen and thawed.

Press juice from heads was also a good source of phenolase. The juice from whole *P. setiferus* heads including stomachs had catecholase activity of $C_b$ 19 after removal of insoluble proteins by centrifugation. Juice from heads, excluding stomachs from the same catch of shrimp had an activity of $C_b$ 32. Press juice from the gill area had 72% greater catecholase activity than that from whole heads, while juice from an area including the stomach and heart but excluding the gills had 30% less catecholase activity than that from whole heads.

When fluid was pressed from freshly caught *P. aztecs* heads and frozen and thawed, a large quantity of clotted blood was observed in the mixture. When the clots were removed, homogenized by grinding with sand, centrifuged and dialyzed, the activity of the dialyzate was $C_b$ 58. When the remainder of the juice was similarly treated its activity was found to be $C_b$ 28.

In a further experiment, hearts were removed from approximately 200 freshly caught shrimp (*P. setiferus*) and dispersed in 10 ml. of 0.1 M sodium phosphate buffer (pH 6.0). This preparation was frozen, thawed and fractionated as outlined in Appendix
VII to yield o-dihydric phenolase specific activities of $C_b$ 80 and $D_b$ 92, but had no apparent monophenolase activity.

Other blood-rich areas found were the sinus openings in the pleopods and the periopods, the segments which connect these legs to the shrimp body and between the shell segments. Blood can be removed from the pericardial sinuses of live shrimp (or immediately after death) either by hypodermic syringe or by aspiration, but only small quantities could be obtained from the small shrimp caught in the inland shrimping areas of Louisiana. This blood was slightly yellow when collected, but turned bluish-green upon exposure to air. When left in contact with air or centrifuged, the blood darkened and often turned black. Blood from P. aztecs always blackens during centrifuging unless treated with an antioxidan t or copper binding agent.

Blood samples were collected from six catches of shrimp and fractionated by various means before being analyzed for phenolase activities. In some instances a small centrifuge was used on the boat to fractionate the blood. After centrifuging (800 X g., 15 minutes) the sediment contained numerous mononucleated leucocytes, and also a few cell fragments which resembled mammalian blood platelets. The nuclei of the former stain dark purple with Giemsa and Wright's stain while the cytoplasm was stained light blue. The other cells stained light purple with these stains. The
treatment and methods for fractionating the six samples of blood are outlined in Appendixes VIII-A thru VIII-F, and a summary of o-dihydric phenolase activities of the different fractions given in Table V. Very little monohydric phenolase activity could be demonstrated in the "plasma" and "serum" fractions of these samples, but several of the "sediment" samples catalyzed the oxidation of L-tyrosine*.

Another sample of P. setiferus blood was fractionated with ammonium sulfate and a globulin fraction obtained which catalyzed the oxidation of pyrocatechol and L-dopa (30 and 5 $\mu$1$\text{O}_2$ uptake per hour, respectively) but not the oxidation of p-cresol, L-tyrosine, p-phenylenediamine or hydroquinone. This fraction was light green and did not darken appreciably when stored overnight at 4$^\circ$ C. Its copper content was 0.08%, and its other properties similar to those of hemocyanin.

The idea of an active phenolase in blood leucocytes was further substantiated by certain properties of shrimp protein exhibited during fractionation. Freezing and thawing, as well as lyophilization tended to increase the total catecholase activity of extracts from shrimp heads. High speed centrifuging of pressed

*Demonstrated qualitatively by a colorimetric method similar to that used for determination of dopa oxidase activity; L-tyrosine was substituted for DL-dopa.
<table>
<thead>
<tr>
<th>Blood Fraction Analyzed</th>
<th>Appendix No.</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Plasma&quot;</td>
<td>&quot;Serum&quot;</td>
<td>&quot;Sediment&quot;</td>
</tr>
<tr>
<td><strong>P. aztecs Shrimp</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cb 91</td>
<td>Cb 116</td>
<td>VIII-A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Frozen before fractionating, no anticoagulant.</td>
</tr>
<tr>
<td>Cb 36</td>
<td>Cb 155</td>
<td>VIII-B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Centrifuged before freezing, no anticoagulant.</td>
</tr>
<tr>
<td>Db 13</td>
<td>Db 70</td>
<td></td>
</tr>
<tr>
<td>Cb 62</td>
<td>Cb 152</td>
<td>VIII-C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Filtered through Celite on fritted glass before freezing, heparin used as anticoagulant. NaHSO₃ used as antioxidant.</td>
</tr>
<tr>
<td>Db 21</td>
<td>Db 39</td>
<td></td>
</tr>
<tr>
<td><strong>P. setiferus Shrimp</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cb 58</td>
<td>Cb 92</td>
<td>VIII-D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Centrifuged before freezing, no anticoagulant.</td>
</tr>
<tr>
<td>Db 13</td>
<td>Db 121</td>
<td></td>
</tr>
<tr>
<td>Cb 55</td>
<td>Cb 34</td>
<td>VIII-E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Centrifuged before freezing, oxalate as anticoagulant.</td>
</tr>
<tr>
<td><strong>Mixture of P. aztecs and P. setiferus Shrimp</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cb 78</td>
<td>Cb 53</td>
<td>VIII-F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Centrifuged before freezing, oxalate as anticoagulant.</td>
</tr>
</tbody>
</table>

*M* Predominantly **P. setiferus Shrimp**
juices left a gel-like residue layered over the bulk of the insoluble pro-
tein which had a higher specific activity than the remainder of the pro-
tein in the sediment or the supernatant.

The presence of an active phenolase in leucocytes rather than
in plasma of blood would explain why blood does not darken in live
shrimp. The possibility exists that the active enzyme is separated
from substrate by the cell wall of the leucocyte which is not perme-
able to the substrate. Disintegration of the corpuscles when bleeding
occurs would release enzyme resulting in darkening of blood.

This, however, does not explain why shell and adhering cuticle
remain undarkened in live shrimp, since both enzyme and substrate
appear to be available in these areas. The prevention of melanosis in
living shrimp might possibly be due to a pyridine nucleotide quinone
reductase and reduced DPN present in shrimp. Since there is a rapid
oxidation of reduced TPN by the enzymatically produced oxidation
products of catechol and dopa (6), such a reducing system may exist.
Dennell (17) found that the redox potential of blood from larvae of the
fly Sarcophaga falcata steadily decreased prior to pupation and then
increased sharply, and melanization occurred. He supposed that in-
hibition of melanization in mature larvae was caused by a dehydro-
genase system which increased the reducing power of the blood.
I. Conclusions

Results presented in this chapter signify that certain oxidases in shrimp tissue and blood are phenolases: copper was necessary for catecholase activity; this activity was reduced by substances which inhibit other phenolase systems; heating at low temperatures increased catecholase activity similar to certain procatecholases previously reported in the literature; low energies of activation indicated that catecholase and dopa oxidase present are highly active and similar to aerobic dehydrogenases from other sources, but dissimilar to anaerobic dehydrogenase systems.

Studies of substrate specificity and anatomical locations of these phenolase(s) indicated that material in abdomen shells with adhering epicuticle, antennae, press juice from heads and blood catalyzed the oxidation of both o-dihydric and monophenols. The shells with attached epicuticle were more active as o-dihydric phenolase while the press juice had greater monophenolase activity. Dopa oxidase in blood and press juice was stereospecific for the L-isomer.

Whole, undialyzed blood catalyzed the oxidation of both mono- and o-dihydric phenols and appeared to contain two distinct phenolases. Hemocyanin apparently can act as a catecholase but a more active o-dihydric phenolase and a monophenolase seem to be produced in the leucocytes or other blood cells. This material is apparently released.
to the serum after disintegration of the cells and could not be separated from hemocyanin since both were globulins.

Tyrosine was present in blood and press juices and probably is a natural substrate for shrimp phenolase. Phenolic compounds attached to protein may also be oxidized in the presence of shrimp phenolase. The sequence of melanophoric changes produced during the blackening of shrimp blood and extracts is exactly that described for the production of "dopa melanins".
SUMMARY

Several physical and chemical properties of the enzyme(s) which cause black spot in shrimp associate them with a group of enzymes generally referred to as phenolases. This enzyme system catalyzed the oxidation of the monohydric and o-dihydric phenols usually identified with phenolase activity of enzymes from different sources.

The necessity of copper-containing protein for the enzymatic oxidation of pyrocatechol was demonstrated and it was also shown that substances known to inhibit activity of phenolases from other sources also inhibited oxidation of pyrocatechol in the presence of shrimp protein. Inhibition by copper-binding substances was further evidence that copper is essential for reactivity of this enzyme system. Other inhibitors such as reducing agents, certain carboxy acids and phenolic complexing agents also inhibited o-dihydric phenolase activity of shrimp fractions.

Methods similar to those used in the purification of mushroom, potato and mammalian catecholase were useful in the purification of shrimp catecholase. These enzyme(s) appeared to be water soluble globulins, not precipitated by 30% saturated ammonium sulfate, but precipitated with 60% saturated ammonium sulfate.
Various blood fractions were very high in o-dihydric phenolase activity. The most active fractions on a protein basis were those found in sediment after blood was centrifuged, and this supports the belief that leucocytes contain a very active phenolase released upon exposure of blood to air. Evidence is also given which indicates that shrimp hemocyanin acts as a catecholase and consequently may be involved in black spot formation.

Catecholase activity was slowly destroyed by heating at $35^\circ C$, but heating for short periods at $50^\circ C$ or slightly higher increased its activity. Part of the enzyme may be present as a proenzyme whose active centers are uncovered by heating. In this respect the shrimp enzyme is similar to other phenolases since the zymogen nature of phenolases from several sources is well known. Heating also could have ruptured leucocytes present to release catecholase, although this was not the only factor involved since heating in the absence of leucocytes also resulted in increased activity of catecholase. Heating for short periods above $65^\circ C$ inactivated the enzyme, and it was almost completely inactivated by heating for 1 minute at $80^\circ C$. Its half-life at $35^\circ C$ was approximately 1 hour.

The activation energy found for catecholase activity identified the enzyme as an aerobic dehydrogenase, and compared favorably with values determined for catecholase activities of phenolases from other sources.
Michaelis constants were determined, but these were not comparable to values obtained by other investigators for phenolase activity possibly because of differences in the methods used to determine activities.

The only natural phenolase substrates identified in shrimp were tyrosine and dopa. The identification of 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) and the possible identification of 5,6-dihydroxy indole as intermediates classified the melanin produced in black spot as "dopa melanin". It was pointed out that the possibility also exists that phenolic groups attached to protein were oxidized to melanins in some dialyzed samples.
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Ed. by S. P. Colowick and N. O. Kaplan, 
Milton Edward Bailey, son of Mr. and Mrs. Silas Edward Bailey, was born at Shreveport, Louisiana on June 7, 1924. He was educated in the public schools of De Ridder, Louisiana, and graduated from De Ridder High School in 1941. He entered the Army Air Force in June, 1943 where he served until he was discharged in February, 1946. In June of the same year he enrolled at the Tulane University of Louisiana and was graduated from that institution in June, 1949 with a Bachelor of Science Degree in Psychology. In June, 1950 he entered Louisiana State University as a graduate student and was a graduate assistant in the Department of Agricultural Chemistry and Biochemistry from July, 1951 to June, 1953, when he completed the requirements for the Master of Science Degree. He has been a full-time Research Assistant in the Department of Agricultural Chemistry and Biochemistry since June, 1953 and is now a candidate for the Degree of Doctor of Philosophy.
APPENDIXES
APPENDIX I

Fresh \textit{P. aztecu}s heads were frozen (-40° F.) and stored for 2 months at this temperature in Marathon cartons. These heads were used to partially purify phenolase as follows: the heads were blended in an equal weight of distilled water and the resulting solution strained through 5 layers of cheese cloth to remove shell and other insoluble material. This extract was then partially frozen and the soft center removed for further purification. It was completely frozen, thawed and centrifuged (5,400 \times \text{g}, 20 \text{ min.}, 4° \text{C.}), and the supernatant (A) filtered through a 1/4 inch pad of celite filter aid (L-665). The filtrate (B) was lyophilized and the dried material ground to a fine powder (Bp) with mortar and pestle. A 5% dispersion of this powder was made by grinding to a paste with 5 ml. distilled water and diluting to 95 ml. This dispersion was again centrifuged (5, 400 \times \text{g}, 10 \text{ min.}, 4° \text{C.}) and the supernatant (C) used to obtain data reported in experiment 1 of Table I. The supernatant was dialyzed (see Appendix II) and the dialyzate used in experiment 2 of Table I.
APPENDIX II

Dialysis was carried out at approximately 3° C. in cellulose dialyzing tubing 21 mm. in diameter. The sample-containing dialysis bags were weighted with glass stoppers and immersed in two liters of cold copper-free water and the dialyzing water stirred by means of an electric stirrer. The dialyzing water was frequently changed and the samples agitated to insure rapid transfer of diffusible ions. A 60% saturated ammonium sulfate solution was practically free of ammonia (Nessler's reagent (60) after dialyzing 48 hours by this method. In the absence of added salts, dialysis time was shortened to 36 hours.
APPENDIX III

The best procedure for estimating protein in determining specific activities of the various enzyme samples was the biuret method of Hiller et al. (63). This method involved the precipitation of protein with trichloroacetic acid, dissolving the precipitate in sodium hydroxide, followed by the addition of copper ions to give a violet color which was compared with a biuret standard solution equivalent to 10 mg. of ovalbumin. The procedure was very useful in determining protein in shrimp tissue extracts since pigments present were precipitated and did not interfere with color absorbance measurements. Results obtained by this method for determining quantities of protein less than 1.5 mg. were inconsistent, and in certain instances when sample quantities were limited the sulfosalicylic acid method of Exton (64) was used and the values obtained converted to equivalent quantities of biuret protein (protein determined by the biuret method).

These two methods were not equivalent for measuring various shrimp proteins when ovalbumin was used as a standard, and a conversion factor was needed to correlate the protein quantities determined by the two methods. The following results (Table VI) were obtained for quantities of protein by the two methods during the analysis of several shrimp samples:
TABLE VI
CORRELATION BETWEEN THE BIURET (63) AND SULFOSALICYLIC ACID (64) METHODS FOR SHRIMP PROTEIN DETERMINATIONS

<table>
<thead>
<tr>
<th>Source of Sample</th>
<th>Sample No.</th>
<th>Protein Biuret (mg./ml.)</th>
<th>Protein S.S.A. (mg./ml.)</th>
<th>Biuret P. S.S.A.P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. setiferus Blood</td>
<td>1.</td>
<td>8.0</td>
<td>12.3</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>2.</td>
<td>7.1</td>
<td>11.0</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>3.</td>
<td>3.8</td>
<td>7.7</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>4.</td>
<td>2.3</td>
<td>3.8</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>5.</td>
<td>3.1</td>
<td>5.7</td>
<td>0.55</td>
</tr>
<tr>
<td>Lyophilized P. setiferus</td>
<td>6.</td>
<td>7.6</td>
<td>11.7</td>
<td>0.65</td>
</tr>
<tr>
<td>head press juice</td>
<td>7.</td>
<td>1.7</td>
<td>2.6</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Samples 1 and 2 were from two different shrimp catches. Blood was taken from pericardial sinuses, frozen and stored 3 days at 10° F., thawed and diluted 1/2. These two samples were centrifuged (5,400 X g., 10 min., 4° C.) and the supernatants (samples 3 and 4 respectively analyzed.

Sample 5 was obtained by dispersing the residue from sample 2 in water.

Samples 6 and 7 were made from lyophilized powder (B_p) in Appendix I by grinding in water and centrifuging (5,400 X g., 10 min., 4° C.). The supernatant fractions were used in the analyses.
APPENDIX IV

A 2% dispersion of lyophilized powder (Bp) of Appendix I was fractionated with ammonium sulfate as follows:

\[
\begin{align*}
\text{2\% dispersion} & \quad \text{centrifuged}\ast \\
\text{residue} & \quad \text{(discarded)} \\
\text{30\% saturated} & \quad (\text{NH}_4)_2\text{SO}_4 \\
\text{precipitate} & \quad \text{supernatant(D)} \\
\text{dissolved} & \quad \text{in water} \\
\text{centrifuged}\ast & \\
\text{residue} & \quad \text{(discarded)} \\
\text{60\% saturated} & \quad (\text{NH}_4)_2\text{SO}_4 \\
\text{precipitate} & \quad \text{supernatant} \\
\text{dissolved} & \quad \text{in water} \\
\text{centrifuged}\ast & \\
\text{residue} & \quad \text{(discarded)} \\
\text{100\% saturated} & \quad (\text{NH}_4)_2\text{SO}_4 \\
\text{precipitate} & \quad \text{supernatant} \\
\text{dissolved} & \quad \text{in water} \\
\text{centrifuged}\ast & \\
\text{residue} & \quad \text{(discarded)} \\
\text{supernatant} & \quad \text{(E)} \\
\text{supernatant} & \quad \text{(F)} \\
\text{supernatant} & \quad \text{(G)}
\end{align*}
\]

* Sample Centrifuged \(1,600 \times g\), 10 min., \(4^\circ F\).
APPENDIX IV CONTINUED

Samples A thru C Appendix I and samples D thru G in the above flow sheet were used to correlate the non-dialyzable dry solids with protein obtained by the Biuret method. The results of these determinations in these samples are given in Table VII.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Dry Weight (mg./ml.)</th>
<th>Biuret Protein (mg./ml.)</th>
<th>Dry Weight Biuret Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>73.5</td>
<td>22.6</td>
<td>3.3</td>
</tr>
<tr>
<td>B</td>
<td>70.0</td>
<td>22.0</td>
<td>3.2</td>
</tr>
<tr>
<td>C</td>
<td>42.0</td>
<td>10.0</td>
<td>4.2</td>
</tr>
<tr>
<td>D</td>
<td>12.2</td>
<td>4.6</td>
<td>2.7</td>
</tr>
<tr>
<td>E</td>
<td>4.2</td>
<td>1.4</td>
<td>3.0</td>
</tr>
<tr>
<td>F</td>
<td>6.7</td>
<td>2.4</td>
<td>2.8</td>
</tr>
<tr>
<td>G</td>
<td>2.7</td>
<td>0.9</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>3.1</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX V

The juices were pressed from shrimp heads in a stainless steel press as described by J. F. Puncochar (personal communication with Dr. E. A. Fieger) U. S. Fish and Wildlife Service, Lockwood Basin, East Boston, Mass. The press core, fitted with a piston, was cylindrical in shape and tapered to a cone containing a drain. Samples were placed in the cylinder, the piston inserted and weights added. The samples were forced into the restricted area of the cone which pressed juices (blood, lymph, cellular constituents, etc.) from the tissues.

Juices obtained in this manner were used for several studies described in the text, and unless otherwise specified were frozen (-40° C.) and thawed several times and centrifuged (5,400 X g., 10 min., 4° C.) and the supernatant used for the various studies.
APPENDIX VI

The eyes, including stalks, were removed from shrimp heads in these studies since the pigment in eyes discolor extracting solutions and because of the possible existence of an inhibitor hormone in the sinus glands of stalks.

Fractionation Scheme I

Eyeless *P. setiferus* heads were blended with 1 liter of cold distilled water for 10 minutes in a Waring blender. This mixture was chilled and centrifuged (800 X g., 10 min., 0°C.). The supernatant solution had a specific activity of C_W 3. This was frozen, thawed and centrifuged (3,500 X g., 20 min., 0°C.). The residue had but slight activity and was discarded, while the supernatant had a specific activity of C_W 4. This was lyophilized and a 5 % (w/v) dispersion made by grinding the powder with sand and water. The suspension was centrifuged (3,500 X g., 20 min., 0°C.) and the supernatant solution had a specific activity of C_W 7 which was further purified by adding 0.03 ml. saturated lead subacetate per ml. of sample and centrifuging (3,500 X g., 20 min., 0°C.). The supernatant had an activity of C_W 10. This was slowly added to 2 volumes of cold (-13°C.) acetone, kept at this temperature for two hours and finally filtered through a Buchner funnel at the same temperature. The precipitate was not allowed to dry until washed several times with cold acetone. The bulk of the acetone was removed by suction and the filter cake dried over sulfuric acid in a vacuum desiccator. The dried material was ground to a fine powder, dispersed in 75 ml. cold 2 % sodium carbonate, dialyzed against distilled water for 24 hours and centrifuged (2,500 X g., 10 min., 0°C.). The specific activity of the supernatant was C_W 33 (C_b 83) and represented a 10-fold increase in purity over the original supernatant solution. The protein in the remainder of the samples was determined by the biuret method because of the small volume of solution.

An equal volume of 0.5 M calcium chloride was added to the last fraction and this was followed by the addition of 0.1 volume of 0.5 M dibasic sodium phosphate. The calcium phosphate gel formed was thoroughly mixed with the sample and kept at 0°C.
overnight. After centrifuging (1,200 X g., 5 min., 0° C.) the supernatant had little activity and was discarded. The protein was then eluted from the phosphate gel by thoroughly stirring with 0.2 M dibasic sodium phosphate and holding for 48 hours with occasional shaking. The sample was then centrifuged (1,200 X g., 5 min., 0° C.), the pH adjusted to 6.0 with 0.2 M monobasic sodium phosphate and the catecholase activity determined. The solution was low in protein and total activity but had a specific activity of \( C_b \) 130. This represented a 17-fold increase in purity over the original supernatant. The volume of this solution was so small that no further purification was attempted.

**Fractionation Scheme II**

Another sample of eyeless heads from the same catch of *P. setiferus* used in scheme I was frozen at -13° C. and blended in a Waring blender. This mixture was added slowly to acetone (-40° C.) and the hard frozen lumps of tissue crushed in a canvas bag and ground in an omnimixer (Servall) kept cold with dry ice and acetone. This was filtered by suction (-13° C.) and the resulting cake washed several times with small portions of cold (-13° C.) acetone and finally dried in vacuum over sulfuric acid. The dried material was then suspended in 2 liters of cold 2% sodium carbonate and again frozen at -13° C., thawed at room temperature and finally centrifuged (2,500 X g., 5 min., 0° C.). The supernatant was dialyzed 24 hours against distilled water. The dialyze had a specific activity of \( C_w \) 14. This was then made 30% saturated with ammonium sulfate, kept for 1 hour at 4° C., centrifuged and the precipitate discarded. The supernatant had a specific activity of \( C_w \) 18. It was again precipitated by adding 0.3 g./ml. of calcium acetate and centrifuged (2,500 X g., 5 min., 0° C.). The supernatant had a specific activity of \( C_w \) 23. This was dialyzed and added to 0.1 volume alumina gel (83), kept at 4° C. for 1 hour and centrifuged (1,500 X g., 5 min., 0° C.). The low active supernatant was discarded and the precipitate suspended in 0.2 M dibasic sodium phosphate and mixed occasionally while kept at 4° C. for
12 hours. This was centrifuged (1,500 X g., 5 min., 0°C.), the pH of the supernatant adjusted to 6.0 with monobasic sodium phosphate. The specific activity of this solution was $C_w$ 33.
APPENDIX VII

**P. setiferus** hearts

- mixed with phosphate buffer (pH 6.0)
- frozen and thawed (x 5)
- ground with sand
- centrifuged (2,500 g, 10 min.)

**supernatant**

- dialyzed (48 hours, 0°C)
- solution turned from light green to dark green

**dialyzate**

- (G_b 80) (D_b 92) darkened during storage at -13°C and turned black after 12 hours at 4°C.

**sediment**

(discarded)
APPENDIX VIII - A

P. aztecus blood

- frozen and thawed
- centrifuged (2,500 g, 10 min.)

"serum" (C\textsubscript{b} 91)

- pH 4.8 acetic acid
- dialyzed
- centrifuged (27,000 g, 30 min.)

- hard, brown ppt.
- dispersed in buffer (pH 6.0)
- dispersed solution (C\textsubscript{b} 3)

- gelatinous ppt.
- extraction with buffer (pH 6.0)
- extract (C\textsubscript{b} 50)

- sediment dispersed in water frozen and thawed (x 2)
- centrifuged (2,500 g, 10 min.)
- supernatant (C\textsubscript{b} 116)

- supernatant (blue green) (C\textsubscript{b} 50)
APPENDIX VIII - B

P. aztecs blood (clotted)

centrifuged (800 g, 15 min.)
(turned black)

"serum"

frozen
thawed

frozen and thawed (x 3)
sonorated

filtered through Celite (L-665)

filtrate
(Cb 36)
(Db 13)

precipitate
ground with sand
extracted with buffer (pH 6.0)
filtered
(Cb 47)

dispersed in 10 ml H2O

frozen
thawed

frozen and thawed (x 3)
sonorated

filtered through Celite (L-665)

filtrate
(Cb 155)
(Db 70)

precipitate
extracted with buffer (pH 6.0)
filtered

filtrate
(Cb 157)
APPENDIX VIII - C

P. aztecs blood

filtered through
Celite and fritted glass

 filtrate

 gelatinous layer
 dispersed in buffer
 (pH 8.0)

 frozen and thawed
 ground with sand
 centrifuged (2,500 g, 10 min.)
 supernatant

dialyzed

dialyzate

(Cb 62)
(Db 21)

(dialyzate

(Cb 70)
(Db 40)

filter pad washed
with buffer (pH 8.0)

filtered through
fritted glass

(dialyzate

(Cb 152)
(Db 39)
APPENDIX VIII - D

P. setiferus blood

(clotted)

centrifuged (800 g, 15 min.)

"serum"

frozen and thawed

mixed with 1 vol. H₂O

filtered through Celite (L-665)

dialyzed

dialyzate

(Cₜ 58)

(Dₜ 13)

sediment

frozen and thawed

ground with sand

filtered through Celite (L-665)

filtrate

dialyzed

dialyzate

(Cₜ 92)

(Dₜ 121)
APPENDIX VIII - E

P. setiferus blood
- oxalate added
- centrifuged (800 g, 15 min.)

"plasma"
- frozen and thawed
- centrifuged (2,500 g, 10 min.)

- supernatant
- sediment
- dialyzed
- dispersed in buffer (pH 6.0)
- dialyzed
- dialyzate (C_b 55)

- supernatant
- sediment
- sonorated
- ground with sand
- dialyzed
- centrifuged (2,500 g, 10 min.)
- supernatant
- extracted with buffer (pH 6.0)
- filtered
- supernatant (C_b 25)
- residue (discarded)
Mixture *P. setiferus* and *P. aztecus* blood oxalate added centrifuged (800 g, 30 min.)

"plasma"

frozen and thawed
dialyzed
dialyzate *(C_b 78)*

sediment

frozen and thawed

sonorated

ground with sand

centrifuged

supernatant

dialyzed
dialyzate *(C_b 53)*

sediment (discarded)
EXAMINATION AND THESIS REPORT

Candidate: Milton E. Bailey

Major Field: Biochemistry

Title of Thesis: BIOCHEMISTRY OF MELANIN FORMATION IN SHRIMP

Approved:

[Signatures and names of major professor, chairman, and dean of the graduate school]

EXAMINING COMMITTEE:

[Signatures of examining committee members]

Date of Examination: April 24, 1958