The tyrosinases of *Fundulus heteroclitus* at different stages of embryonic development

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The study described here was undertaken in an effort to determine whether multiple forms of tyrosinase (EC 1.10.3.1) are present in *Fundulus heteroclitus* during embryonic development. If they are present, do they appear simultaneously in the course of development and do they continue to be produced and maintained without change during the part of the life cycle when tyrosinase should normally be active? Multiple forms of tyrosinase have been found in *Neurospora crassa* (Sussman, 1961; Fox & Burnett, 1962; Fling, Horowitz & Heinemann, 1963), mushroom (Smith & Kruger, 1962; Bouchilloux, McMihill & Mason, 1963; Jolley & Mason, 1965), potato (Patil & Zucker, 1965), *Drosophila melanogaster* (Lewis & Lewis, 1963; Mitchell & Weber, 1965), the eggs of *Rana pipiens* (Turney, 1964), goldfish (Kim & Tchen, 1962; Kim, Tchen & Chavin, 1962), hamster melanoma (Pomerantz, 1963, 1966) and mouse melanoma (Shimao, 1962; Burnett, Seiler & Brown, 1967). By using a variety of physical and chemical techniques to isolate the tyrosinases present in various organisms and organs, the investigators cited above have been able to establish numerous specific characteristics of the enzymes. Their published data suggest that in both plants and animals there may be variation in the chemical and physical characteristics of individual, enzymically active tyrosinase molecules. Experiments have not yet revealed, however, the metabolic or functional significance of the various forms of an enzyme within a single system. Since the presence of multiple forms of tyrosinase within a single system seems to be universal, it was anticipated from the outset that multiple forms of tyrosinase would be found in the embryos of *Fundulus heteroclitus*.

The formation of melanin is catalysed in both plants and animals by the enzyme tyrosinase. In the presence of this enzyme, tyrosine is oxidized to 3,4-dihydroxyphenylalanine (DOPA) and DOPA, in turn, is oxidized to DOPA-quinone. The conversion of DOPA-quinone to melanin proceeds in several steps (Lerner & Fitzpatrick, 1950).

The presence of multiple forms of tyrosinase within a single organism or organ could be the result of chance mutations. Such mutations might cause subtle

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changes that would not significantly affect the metabolic function of the enzyme. If, however, two or more forms of tyrosinase were characteristically to become active at different stages of development and thereafter remain independent, one might infer that there are functional differences between the various forms of the enzyme.

**MATERIALS AND METHODS**

*Fundulus heteroclitus* was chosen for study because manifestations of tyrosinase activity, namely the appearance and accumulation of the pigment melanin, are easily discernible during its early embryonic development and continue to be evident throughout larval life. The specimens used in this study were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, during the spawning season (June through August). Ova and milt were expressed from females and males and mixed in sea-water. When milt had been in contact with the eggs for 30 min, the water was changed and the eggs were allowed to stand in milt-free sea-water at room temperature (22 °C) for 1–2 h. At the end of this period, the eggs were rolled on paper towelling to remove their outer coat, which hampers observation of development. The developing zygotes were maintained in sea-water at room temperature. At the various stages of development (Armstrong & Child, 1965) listed in Table 1, samples of approximately 300 were removed for analysis. During the later stages of embryonic development (stages 29–31), pigment is recognizable in two major locales: in the retinal pigment epithelium and in melanophores scattered throughout the body tissues. At stages 29, 31 and 33 two samples of 300 zygotes were removed so that the tyrosinase activity from intact embryos could be compared with that from embryos from which the eyes had been removed. Apart from this, no attempt

### Table 1. Characterization of Fundulus heteroclitus at various stages of embryonic development at room temperature (22 °C)

<table>
<thead>
<tr>
<th>Stage of development*</th>
<th>Age of embryo (h)</th>
<th>Major identifying characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>56</td>
<td>Main divisions of the brain are differentiated and optic lobes are prominent</td>
</tr>
<tr>
<td>23</td>
<td>66</td>
<td>Integumentary melanophores are first visible</td>
</tr>
<tr>
<td>25</td>
<td>84</td>
<td>Circulation begins</td>
</tr>
<tr>
<td>27</td>
<td>112</td>
<td>Dendrites of melanophores are clearly visible</td>
</tr>
<tr>
<td>29†</td>
<td>144</td>
<td>Eyes begin to be pigmented</td>
</tr>
<tr>
<td>31†</td>
<td>168</td>
<td>Heart chambers are differentiated</td>
</tr>
<tr>
<td>33†</td>
<td>216</td>
<td>Ectoderm of the yolk sac is detached anteriorly at the lower level of the forebrain</td>
</tr>
<tr>
<td>34</td>
<td>228</td>
<td>Fish has hatched</td>
</tr>
<tr>
<td>39</td>
<td>384</td>
<td>Yolk sac is completely absorbed</td>
</tr>
</tbody>
</table>

* See Armstrong & Child (1965).
† Stages at which eyes were removed from duplicate samples for comparison.
Table 2. Isolation and fractionation of tyrosinase from Fundulus heteroclitus

Unless otherwise specified, all procedures were carried out at 5 °C and all dialysis was performed against 1 L of 0.05 M tris-glycine buffer, pH 8.2.

300 EMBRYOS
(In 2 ml of 0.05 M tris-glycine buffer, pH 8.2, containing 0.25 M sucrose)
| Homogenize for 10 min
| Dialyse for 12 h
| Centrifuge for 20 min at 35000 g

SUPERNATANT (S₁)

PELLET (P₁)

Add 1/10 vol. saturated (NH₄)₂SO₄ and 1 vol. of ice-cold acetone equal to the vol. of the supernatant plus the (NH₄)₂SO₄
| Let stand for 1 h at -20 °C
| Centrifuge for 20 min at 35000 g

SUPERNATANT (S₂)

PELLET (P₂)

Add 0.2 g sucrose

Fraction I

Soluble tyrosinase
(T₁, T₂)

PELLET (P₃)

Suspend in 1 ml of tris-glycine buffer containing 6 M urea
| Homogenize for 5 min
| Add 5 ml ice-cold acetone
| Let stand for 30 min at -20 °C
| Centrifuge for 20 min at 35000 g

SUPERNATANT (S₄)

PELLET (P₄)

Add 0.2 g sucrose

Fraction II

Solubilized tyrosinase
(T₃, T₂, T₄)
was made to determine the localization of tyrosinase activity in specific parts of the body. Each sample was frozen immediately in a minimum volume of seawater and stored at $-20$ °C until analysed.

Solutions containing active tyrosinase were prepared from the various 300-egg samples by a modification of the procedure of Brown & Ward (1957) (Table 2). It was found impracticable to study fresh homogenates of the various samples because extraneous material interfered with proper band development within the gel. When prepared by the technique outlined in Table 2 fraction I contains soluble enzyme from the initial homogenate; fraction II contains little or no enzyme; and fraction III may contain soluble enzyme that was trapped in the particulate material during sedimentation, or enzyme that had been bound to particulate material and released (solubilized) by the action of urea, or enzyme from both these sources. Duplicate aliquots of each fraction were subjected to acrylamide-gel electrophoresis (Davis, 1964; Ornstein, 1964) at room temperature for 30–60 min at 5 mA/tube. Bromphenol blue, added to the buffer in the cathode reservoir, served as a tracking dye. After electrophoresis the gels were neutralized in 0·1 M phosphate buffer, pH 6·8, for 30 min and then immersed in 0·15% L-DOPA in 0·1 M phosphate buffer, pH 6·8. Since the manifestation of tyrosinase activity in the tubes does not appear for more than 24 h when tyrosinase is the substrate, L-DOPA was routinely used as the substrate in these experiments. The brown bands of pigment (melanin) that form where tyrosinase reacts with L-DOPA serve to indicate the location of the enzyme(s) in the tubes. The $R_x$ value for the enzyme, which is defined as the ratio between the distance traveled by the enzyme into the gel and the distance traveled by the tracking dye, constitutes a means of characterizing the enzyme, while the relative density of the pigment bands produced constitutes a somewhat quantitative measure of the amount of enzyme present.

RESULTS

The presence of tyrosinase activity was first seen as two very faint brown bands in fraction I (the soluble fraction) at stage 23. The tyrosinases responsible for the formation of these bands have $R_x$ values of 0·75 (the more anodic) and 0·64; they are here designated $T^1$ and $T^2$ respectively. With minor variations, this pattern of band formation continued through stage 27; thereafter the bands became much less distinct.

From stage 29 through stage 33, three bands of tyrosinase activity were consistently found in fraction III. The $R_x$ values of the tyrosinase in two of these bands were identical with those of the $T^1$ and $T^2$ from fraction I; that of the least anodic band was 0·31 (Plate 1). Preparations from eyeless embryos were compared with preparations from whole embryos at stages 29, 31 and 33. The enzymic pattern of eyeless embryos did not differ consistently from that of whole embryos.

The amount of tyrosinase activity ($T^1$ and $T^2$) in fraction I decreased steadily
Patterns of the activity of soluble (A) and solubilized (B) tyrosinase derived from *Fundulus heteroclitus* at stage 29 of embryonic development as shown by acrylamide-gel electrophoresis. As oriented here, the enzyme migrated from the bottom (cathode) toward the top (anode) of the gel.

A. *Fraction I.* Rapidly migrating, bromphenol blue tracking dye forms the upper band. The position of this band (front) in the tube provides a basis for calculation of the $R_x$ values of the enzymes. The bands of soluble T1 and T2 are clearly visible below the front.

B. *Fraction III.* The tracking dye (front) is in the same position as in *Fraction I.* The bands of solubilized T1 and T2 are in the same position as those of soluble T1 and T2. A third band, that of solubilized T3, is present below them.
from stage 29 through stage 39 (the last stage studied), as shown by the rate of
development of color and the density of pigment bands within the gel. Con-
currently, the activity of tyrosinase (T1, T2, T3) in fraction III seemed to remain
constant as the age of the embryo increased. Throughout the period of embryonic
development under investigation, no change could be observed in the relative
activity of the two forms of tyrosinase in fraction I or of the three forms in
fraction III.

**DISCUSSION**

The number of fertilized eggs available for these experiments was sufficient
for only one series of enzyme extractions at each stage of development. Results
are consistent, however, although the 300-egg samples provided only enough
enzyme to produce faint bands of pigment.

Work by other investigators (Kim & Tchen, 1962; Kim et al. 1962; Lewis &
Lewis, 1963; Seiji, Shimao, Birbeck & Fitzpatrick, 1963; Chian & Wilgram,
1967) has shown that activators and inhibitors play a role in the functioning of
tyrosinase in other organisms, and it is possible that the synthesis and activation
of tyrosinase during the embryonic development of *Fundulus heteroclitus* may
depend on equally complex mechanisms. Whatever the mechanism by which
active tyrosinase may be formed within this fish, T1 and T2 become active at the
same time in extracts prepared from *F. heteroclitus* during the early stages of
pigment production. This suggests that in vivo there may exist simultaneously
two enzymes, corresponding to T1 and T2 in our preparation, that have identical
schedules of synthesis and activation.

In stage 29 and thereafter, three forms of active tyrosinase could be simul-
taneously extracted from fraction III. It is possible, but not probable, that the
T1 and T2 of this fraction had been trapped during pellet formation in the first
two centrifugations (see Table 2) and later released when the pellets were gently
resuspended. From stage 29 to stage 39 the density of the bands of T1 and T2 of
fraction III remained constant whereas that of the isologous bands of fraction I
gradually decreased. It would appear, therefore, that most of the T1 and T2 of
fraction III was released from particulate material when urea was added.
Since T3 was not detected in fraction I, which contained the easily extractable,
soluble cell components, it is unlikely that T3 is formed *de novo* within the
melanophore. The presence of T3 in fraction III is probably due to the action of
urea on the bonding of this form of the enzyme to particulate material.

Although the interrelationships of these various tyrosinases have not yet been
determined in detail, the data reported here suggest that, as melanin is formed in
living systems, soluble enzymes may become associated with particulate material
(e.g., premelanosomes, melanosomes). This concept is well supported by the
work of Seiji *et al.* (1963). These investigators demonstrated that in the melano-
cytes of B-16 mouse melanoma tyrosinase is synthesized by ‘small granules’
(presumably particles of ribonucleoprotein) and stored in distinctive cytoplasmic
organelles (melanosomes). The enzyme found only in solubilized form may be derived from either of the two soluble enzymes or from a combination of the two. This concept is supported by recent experimental evidence: Burnett et al. (1967) have found that the solubilized tyrosinase of mouse melanoma is a unique, complex enzyme which contains at least four distinct components. In the study described here some of the particle-bound tyrosinase released in soluble form by urea seems to be electrophoretically indistinguishable from tyrosinases that were not particle-bound. The persistence of active forms of tyrosinase in fraction III is consistent with the work of Kim et al. (1962), who found that in the mature goldfish (Carassius auratus L.) the hyperpigmentation-associated stress correlated more closely with an increase in the tyrosinase activity of a particulate fraction than with an increase in the activity of a soluble fraction.

SUMMARY
1. The study described here was undertaken in an effort to determine: (a) whether multiple forms of tyrosinase are present in the embryos of Fundulus heteroclitus at different stages of development; (b) the stages of development at which active tyrosinases first appear in the embryos, and (c) the persistence of the tyrosinases found.

2. The material studied was prepared from developing embryos by differential centrifugation and analysed by acrylamide-gel electrophoresis.

3. Two forms of active soluble tyrosinase (T1, T2) were first detected at stage 23 when pigment cells first became visible in the embryo; they persisted with apparently diminishing activity throughout stage 39, the last stage of development studied.

4. Three forms of active tyrosinase (T1, T2, T3) could be released from particulate material. They were first detected at stage 29 soon after the dendrites of the melanophores became clearly visible. These solubilized tyrosinases continued to be active and appeared to remain unchanged throughout the course of these experiments.

5. Solubilized T1 and T2 appear to be electrophoretically identical with soluble T1 and T2. It is possible that T3 may be derived from T1 or T2 or from the combination of T1 and T2. It is unlikely that T3 is synthesized de novo within the melanophore.

ZUSAMMENFASSUNG
1. Der Grund für die hier beschriebene Untersuchung war, festzustellen: (a) ob die vielfachen Formen der Tyrosinasen in den verschiedenen Stadien des Embryos vom Fundulus heteroclitus vorhanden sind; (b) zu welcher Zeit der Entwicklung des Embryos die aktiven Tyrosinasen auftreten, und (c) die Beständigkeit des Auftretens der gefundenen Tyrosinasen.

2. Das studierte Material war durch ultrazentrifugations Techniken von den
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verschiedenen Stadien befindenden Embryos gewonnen und analysiert mit Acrylamid Gel Elektrophorese.


5. Die lösbar gemachten T¹ und T² sind elektrophoretisch identisch mit den löslichen T¹ und T². Es ist möglich, dass das T³ vom T¹ oder T² oder von der Kombination T¹ und T² entsteht. Es ist unwahrscheinlich, dass das T³ in Melanophor de novo entsteht.

This investigation was supported by United States Public Health Service Research Grants: Grant no. CA-08292 from the National Cancer Institute and Grant no. 5 T 5-GM-1651 from the National Institute of General Medical Sciences.

The authors wish to thank Dr George Szabo, Assistant Professor of Anatomy in the Department of Dermatology, Harvard Medical School, for his interest in this study and for his hospitality in allowing them to use his laboratory at Woods Hole for procedures related to the fertilization of the Fundulus heteroclitus eggs.

Mr Hans Seiler of the Department of Dermatology of the Harvard Medical School has kindly translated the summary of this paper into German.

REFERENCES


(Manuscript received 31 May 1967, revised 11 September 1967)