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AN AUTORADIOGRAPHIC ANALYSIS OF MELANOGENESIS IN THE FIDDLER CRAB, *UCA PUGNAX* (S. I. SMITH)

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The epidermis plays a central role in the metabolic activities of crustacea. One facet of these activities involves pigment cells in which two distinct phases of behavior can be distinguished: (1) physiological color change, a rapid mobilization of pigment leading to pigment migration, probably within pre-established cellular channels, and (2), morphological color change, a slower modification of the quantity of pigment per chromatophore or of chromatophore number per unit area of epidermis (Brown, 1934; Green, 1964a, b). An hypothesis (Babak, 1912; Brown, 1934) which closely relates morphological color change to physiological color change may not be valid for crustacean. Specifically, there seems to be no correlation between the state of pigment dispersal within the chromatophore and melanin synthesis (Green, 1964b).

The present study reports the results of autoradiographic analysis of melanin formation in fiddler crab epidermis.

Material and Methods

Autoradiography at the tissue level

Kukita and Fitzpatrick (1955) and Brunet and Small (1959) prepared autoradiographs for a study of melanogenesis from human and mouse tissue. In both cases tissue was incubated in ^{14}C-tyrosine, which is incorporated into insoluble melanin if the melanocytes are active. Both teams of workers reported difficulty in observing exposed silver grains against the background of melanin. This difficulty is partially obviated with fiddler crab tissue because the pigment is periodically distributed into the finest processes of the melanophore. When so distributed the pigment density is such that melanin granules and silver grains can be distinguished. The use of phase contrast optics also heightens the optical disparity between silver grains and melanin granules. Illumination through a trinocular tube of the microscope is reflected from silver grains and provides an additional aid in distinguishing melanin granules and silver grains.

Male specimens of *Uca pugnax* were collected from the salt marsh at Sippewisset, Massachusetts, and brought to the Marine Biological Laboratory, Woods

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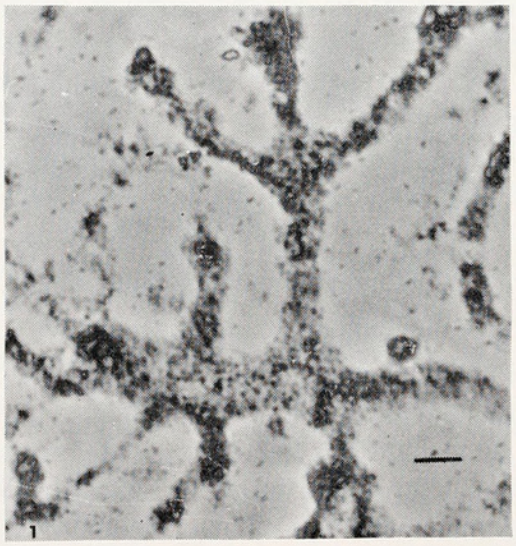
Hole, Massachusetts. The crabs were maintained unfed in a fiberglass tub with perforated floor. Flowing sea water maintained a water level of about $\frac{1}{8}$ inch. Crabs were utilized randomly from this supply.

Entire and destalked animals were injected with 0.05 ml of 2.5 μC 2, 5, -^{3}H-tyrosine in sea water through the arthrodial membrane at the base of the fourth walking leg. All crabs were dechelated to reduce the hemocoel volume, and maintained for two days on a black background to maximize pigment dispersal.

The tissue chosen for examination was the epidermis lining the carapace. This tissue is extremely friable and cannot be stripped from the exoskeleton without considerable destruction and distortion. Consequently the epidermis-exoskeleton complex was fixed *in situ* in San Felice fixative (10% formalin in sea water containing 1% chromic acid plus 10 drops acetic acid). After fixation the hardened epidermis was stripped from the exoskeleton with minimal derangement of the tissue. The epidermis was washed in several changes of distilled water to remove the fixative and free tyrosine.

The epidermis was then spread on gelatin-coated microscope slides (Brunet and Small, 1959). Slides were dipped in Kodak NTB-2 liquid photographic emulsion diluted $\frac{1}{3}$ with distilled water, dried and stored in light-tight boxes in the refrigerator for 35 days. They were developed in Dektol, fixed, washed and



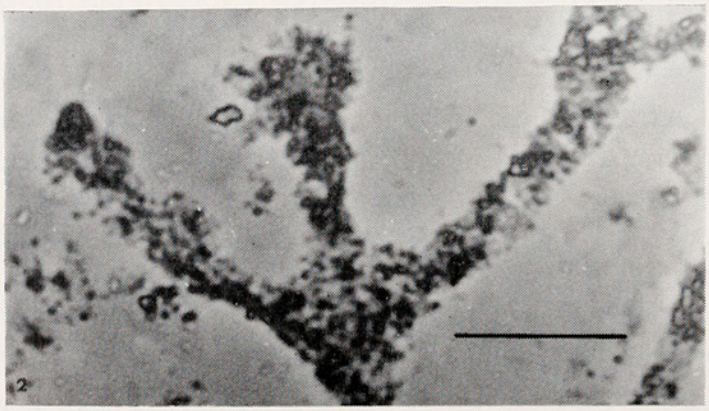


FIGURE 1. Autoradiograph prepared from a whole-mount of fiddler crab epidermis with dispersed pigment; bar = 50 μ; phase contrast optics.

FIGURE 2. Autoradiograph prepared from a whole-mount of fiddler crab epidermis; detail of Figure 1, bar = 50 μ; phase contrast optics.

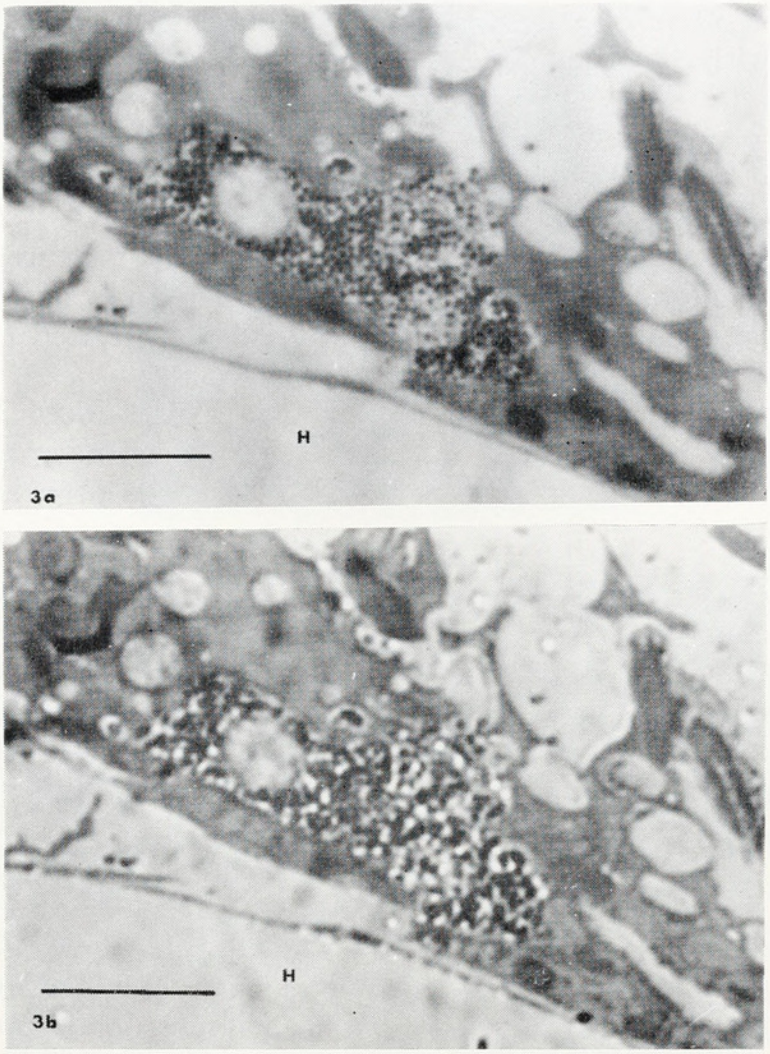


FIGURE 3a. Autoradiograph prepared from 0.5 μ thick section of fiddler crab epidermis, H = hemocoel; phase contrast optics, bar = 20 μ; 3b. As in Figure 3a but epi-illuminated.

dehydrated in a graded series of alcohols. The emulsion-covered tissue was mounted with permount. Observations and photographs were made with Wild phase-optical equipment.

Autoradiography at the cellular level

Additional studies of melanogenesis were made as part of an electron microscope study of the structure of the epidermis. Techniques appropriate for electron microscope observation also yield thick tissue sections suitable for autoradiographic study with the light microscope.

Entire and destalked crabs were each injected with 2.5 μC ^{3}H-tyrosine in 0.05 ml sea water and were maintained as previously described. Crabs destalked two days before injection were maintained for 4 days. The carapace was removed from the crab and freed of adhering tissues (*e.g.*, digestive gland) and was cut into pieces about 5 mm^2. These pieces of exoskeleton with attached epidermis were fixed in 5 ml of 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer containing 0.005 *M* $CaCl_2$ and 0.25 *M* sucrose. After 1.5 hours in the fixative the tissue was washed in 5 ml 0.2 *M* cacodylate buffer for 30 minutes with three changes of buffer. Post-fixation in 1.3% OsO_4 in 0.2 *M* sodium cacodylate buffer for 1 hour

was followed by a 30-minute distilled water wash. The preceding steps were carried out at 0° C. with continuous agitation.

Immediately after the final washing, the tissue was peeled from the exoskeleton and was cut into pieces 1 mm^2. Dehydration in a graded series of cold acetone-H_2O mixtures followed. The tissue remained in 100% acetone at room temperature for 8 minutes with two changes.

Infiltration in a graded series of epon-araldite-acetone mixtures to 100% epon-araldite (1.26:1) followed over a three hour period (Voelz and Dworkin 1962). The tissue was flat-embedded in epon-araldite and polymerized at 60° C. for 72 hours.

Sections 0.3–0.5 μ thick were prepared with a Porter-Blum MT-2 ultratome. Sections were floated on water and attached to pre-cleaned microscope slides.

Autoradiographs were prepared from this material as previously described.

Results

Green, 1964b found that *in vivo* incorporation of ^{14}C-tyrosine into melanin was independent of the degree of dispersal of the pigment granules within the melanophore. This does not show that melanin synthesis occurs within the melanophores. The present study was designed to identify the site of melanin synthesis in *Uca pugnax*. Autoradiographs prepared from the epidermis indicate that melanin synthesis occurs only within the melanophore.

Figure 1 is a low power view of epidermis, containing a melanophore with dispersed pigment. A grid was superimposed over this photograph and the silver grains in 20 randomly chosen squares over a melanophore process were counted. Similarly silver grains located in 20 squares which were not over a melanophore process were counted. Squares outside of the boundary of the melanophore had 2.9 ± 0.5 (mean $\pm$ S.E.) grains per square as compared with 13.75 ± 0.8 grains per square over a portion of a melanophore. These counts differ significantly ($P < 0.01$—Student's t-test).

Figure 2 is a higher power view of part of Figure 1. Silver grains can be distinguished from melanin granules by their hollow centers. The apparent hollow center of the silver grain is due to its light-reflecting characteristic as opposed to the light-absorbing nature of the melanin granule. This disparity may be heightened by illuminating the section through one of the trinocular barrels of the microscope. With such epi-illumination the silver grains reflect light, whereas the melanin granules remain dull (Fig. 3a, b).

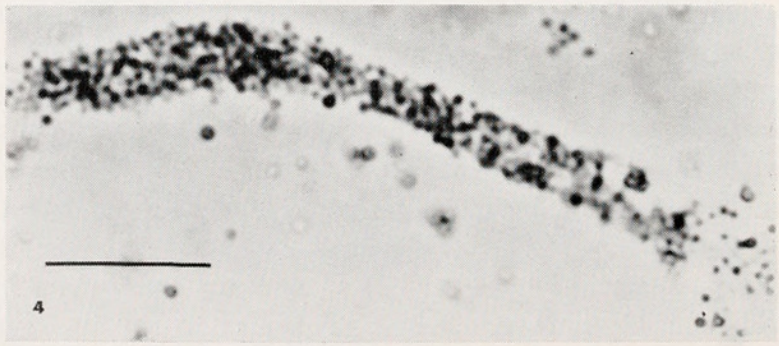
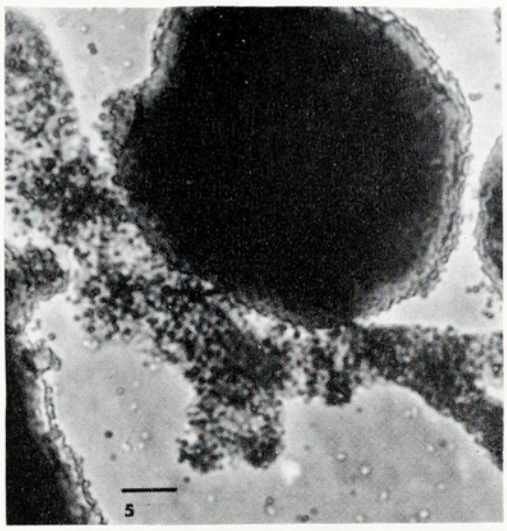


Figure 4. Autoradiograph prepared from a whole-mount of fiddler crab epidermis with dispersed pigment, bar = 20 μ.



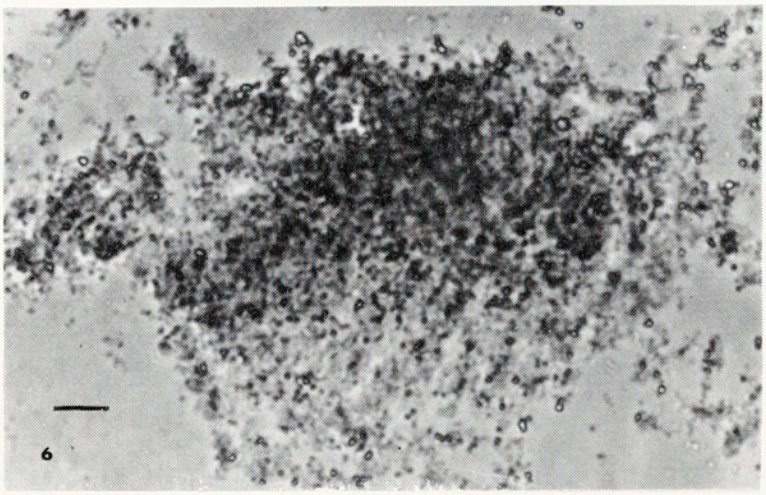


FIGURE 5. Autoradiograph prepared from a whole-mount of destalked fiddler crab epidemis with both dispersed and contracted pigment; upper pigment mass contracted, bar = 20 μ.

FIGURE 6. Autoradiograph prepared from a whole-mount of destalked fiddler crab epidermis. Contracted pigment mass ruptured releasing pigment granules. Lower density of granules enables distinguishing silver grains from melanin granules, bar = 20 μ.

Figure 4 is high-power phase optical view of a melanophore process. This section was analyzed in a fashion similar to Figure 1. Twenty squares not containing any portion of a melanophore had 0.95 ± 0.2 silver grains per square as compared with 4.2 ± 0.4 grains per square over a portion of the melanophore. These grain counts are significantly different ($P < 0.01$).

Crabs whose eye-stalks have been removed have melanin concentrated in the center of the melanophore (Fig. 5). In this condition it is difficult to distinguish silver grains from melanin granules because of the high density of the latter. Only at the edges of the mass of melanin can silver grains be distinguished. In a number of cases preparative treatment of the tissue led to rupture of the concentrated mass of melanin. In these fortuitous cases the density of the melanin granules is reduced and the silver grains can be distinguished (Fig. 6).

In thick sections prepared as for electron microscopy it can be seen that the melanophores are present on both the environmental and hemocoel surfaces of the epidermis (Fig. 7ab). Figure 8 shows a comparable section with the pigment partially dispersed. Tissue with dispersed pigment is considerably more resistant

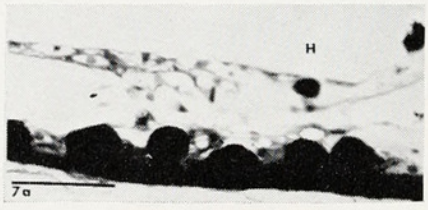
to damage during processing than is tissue with contracted pigment granules. Apparently the filling of cellular channels with melanin confers considerable strength on the tissue (*cf.* Elias 1942).

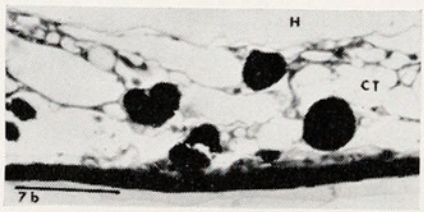
Figures 9 (a–c) and 10 show some characteristics of the finer melanophore processes. Figure 9a shows the confluence of melanophore processes, Figure 9b the termination of a process, and Figure 9c the single-file nature of pigment granule migration within a process. Figure 10 shows melanin granules entering (or leaving) a process.

Melanin occurs in the fiddler crab epidermis in approximately spherical granules with a mean diameter of 0.98 $\mu \pm 0.02$. In comparison, mammalian melanin granules (melanosomes) are 0.1 μ in length and 0.05 μ in width (Drochmans 1963, 1966). Light microscopy reveals no internal structure within *Uca* melanin granules but studies with the electron microscope are being carried out.

Discussion

Kukita and Fitzpatrick (1955) proposed an autoradiographic assay for mammalian tyrosinase activity based on the enzyme-catalyzed conversion of water-soluble ^{14}C- or ^{3}H-tyrosine to water-insoluble melanin. The theoretical scheme for melanin synthesis involves the enzymatic (tyrosinase) oxidation of L-tyrosine to 3,4-dihy-





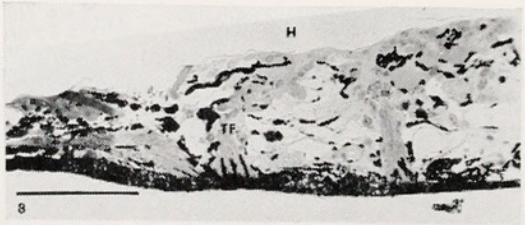


Figure 7a. Thin section (0.5 μ) of fiddler crab epidermis with contracted pigment, H = hemocoel bounded by basement membrane, bar = 50 μ; 7b. As in Figure 7a, note presence of melanophores on both epidermal surfaces and also in the spongy sub-epidermal connective tissue (C. T.), bar = 50 μ.

Figure 8. Thin section (0.5 μ) of fiddler crab epidermis with partially expanded pigment. Note that there is an indication that the melanophores follow the course of tonofibrillae (TF), bar = 100 μ.

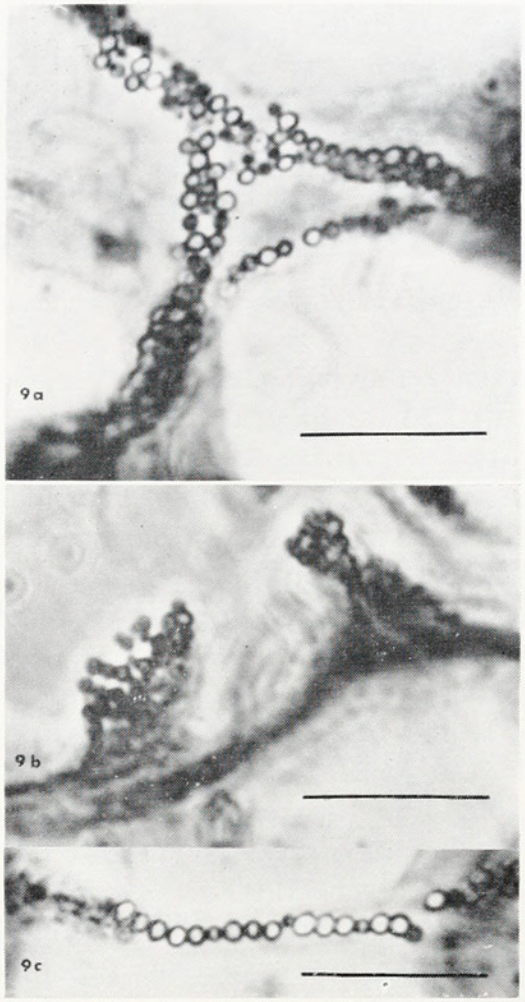


FIGURE 9a. Phase contrast optics, confluence of several melanophore processes, bar = 10 μ; 9b. As in Figure 9a, terminus of melanophore process, bar = 10 μ; 9c. as in Figure 9a, melanin granules in a fine process, bar = 10 μ.

droxyphenylalanine (DOPA) and then to dopaquinone. Dopaquinone undergoes spontaneous oxidation and reduction reactions to yield indole-5,6-quinone. This compound is polymerized to form a large molecule which is thought to be bound through its quinone linkages to the amino or sulfhydral groups of the protein matrix of the pigment granules (Mason, 1959, 1967; Duchon, Fitzpatrick and Seiji, 1968). Nicolaus, Piattelli and Fattorusso (1964) and Nicolaus and Piattelli (1965) (see also Mason, 1967; Duchon *et al.*, 1968) however, hypothesize that melanin is not homopolymeric (a regular polymer of idole-5,6-quinone) but consists of a random polymer (poikilopolymer) of several different monomers including in part, DOPA, indole-5,6-quinone, and 2,3-dihydroxyindole. In either case the product melanin is water-insoluble and has incorporated into it the isotopic label.

Tyrosinase is generally believed to occur in the matrix of the melanosome (Moyer, 1963, Seiji, 1966). Thus the formation of melanin is presumptive evidence for the presence of tyrosinase and melanin granules. In the present work the localization of radioactivity derived from ^{3}H-tyrosine over granules within the melanophore is evidence that melanogenesis occurs only in this cell. Melanogenesis apparently occurs independently of the state of pigment dispersion. The present technique, however, is not sensitive enough to detect short-term differences in the rate of melanin synthesis that may exist between melanophores with expanded

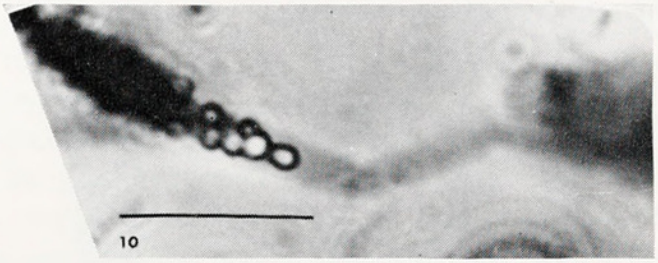


FIGURE 10. As in Figure 9a, melanin granules entering or leaving a process, bar = 10 μ.

pigment and those with contracted pigment. Further work utilizing an *in vitro* assay system will be required to establish this point conclusively.

Seiji, 1966 and Wood and Ingraham, 1965 have suggested as successive layers of melanoprotein accumulate on the melanosome, tyrosinase is inactivated. These workers conclude that there is a reciprocal relationship between degree of melanization and tyrosinase activity within a melanin granule. The present light microscope autoradiographic techniques are not sufficiently sensitive to detect variations in melanogenesis at the sub-cellular level.

The crustacean carapace arises from an epidermal fold of the maxillary segment (Tiegs and Manton, 1958), and thereafter fuses with the united terga of at least 10 body segments behind the head (Snodgrass, 1952). The carapace and its underlying epidermis is folded in the region of the branchial chamber (Travis, 1955, 1957). Figures 7 (ab) and 8 indicate that the epidermis is also double in the region of the back, probably reflecting its origin. As these figures indicate, and as Travis (1955, 1957) showed, for the branchiostegite, the epidermis consists of two layers of cells separated by intra-epidermal (*cf.* subepidermal, Travis, 1955, 1957) connective tissue of a loose spongy type. In *Uca* the connective tissue may be invaded by melanophores (Figs. 7b and 8).

Perkins and Snook (1932) investigated the movement of the red carotenoprotein pigment in chromatophores of the prawn *Palaemonetes*. They found the red pigment to be granular in nature and suggested that the "granules and the protoplasmic medium in which they are carried flow out into collapsed tubes which are the walls of the branched chromatophore, fixed in position, and lying in tissue spaces." Melanin granules in *Uca* are similarly found within chromatophores and seemingly the chromatophores are constructed along the plan proposed by Perkins and Snook. No information is presently available concerning the mechanism of pigment migration in crustacean chromatophores.

I would like to acknowledge the able assistance of Mr. T. Richard Nichols, Miss Mary Rose Neff, and Miss Emily Paynter.

SUMMARY

1. Autoradiographs prepared from whole mounts and sections of *Uca pugnax* epidermis labelled *in vivo* with ^{3}H-tyrosine showed incorporation only into melanin granules within melanophores.

2. Incorporation occurred whether the pigment was in a concentrated (*e.g.*, destalked crabs) or expanded (*e.g.*, entire crabs—black background) condition.

3. Fiddler crab melanin granules appear spherical in light micrographs and have a mean diameter ($\pm$S.E.) of 0.98 μ $\pm$0.02.

4. The epidermis of the back of the fiddler crab shows two layers of melanophores, one lining the hemocoel basement membrane, the other lining the exoskeleton. This may be a result of the formation of the carapace as a fold from the maxillary segment. Melanophores and melanophore processes also are found in the spongy intra-epidermal connective tissue.

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