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Analysis of Red and Yellow Pigments in Two Mutants Of the Siamese Fighting Fish, Betta splendens

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Analysis of Red and Yellow Pigments in Two Mutants Of the Siamese Fighting Fish, *Betta splendens*[1]

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SYNOPSIS: Red and yellow pigments of two mutants of the Siamese Fighting Fish, *Betta splendens*, were analyzed. Chromatographic separations of integumentary material showed that red mutants have red pterin and carotenoid pigments. Yellow mutants have both types but not the same specific pigments.

INDEX DESCRIPTORS: Fish pigments, carotenoids, pterins, Siamese Fighting Fish, *Betta splendens*.

Among the several color mutations in the Siamese Fighting Fish, *Betta splendens*, are several which affect red and yellow color expression. Scant information was offered by Goodrich, Hill and Arrick (1941). They reported red pigment to be erythropterin and yellow, lutein. They noted a limitation of the red to fin areas on light colored (cambodia) phenotypes.

Subsequent studies by Lucas (1968), revealed genetic evidence of more complex relationships between red and yellow pigmentary elements. A "non-red" mutation was discovered which caused the fish to appear yellow in areas where they normally would have been red. It was suspected that the non-red phenotypes resulted from a block in the biosynthetic pathway responsible for the formation of red pigment.

This study attempted to confirm the presence of lutein (yellow) and erythropterin (red) in Bettas as reported by Goodrich, et al., and to provide additional and more precise information for genetic purposes.

MATERIALS AND METHODS

To show the presence (or absence) of carotenoid and/or pterin pigments in red and yellow mutant stocks of Bettas, two analytical methods were employed:

(1) Modifications of procedures used by Sumner and Fox as outlined by Goodrich, et al. (1941) using different solvents to extract the various pigments, and

(2) Modifications of methods of
   (a.) pterin analysis, as outlined by D. Fox (1953) and Fox & Vevers (1960), and
   (b.) carotenoid analysis as outlined by Goodwin (1955).

Various chemical and physical analytical techniques were used, including determination of visible and ultraviolet spectra, column and thin layer chromatography and comparison of fluorescent properties. Tentative identification was made by chromatographic separation and comparisons with previously identified substances in other organisms. Plant pigments were used as reference standards for the carotenoids (Goodwin, 1955), Drosophila eye pigments for the pterins (Hadorn, 1962).

Silica gel and alumina (aluminum oxide) were the adsorbents used for both TLC and column chromatography. The solvents, or elutants, varied depending on the polarity needed to dissolve and move a sample's components. Adsorbent and solvents were selected with reference to those used by Goodwin (1955) for carotenoid analysis and Hadorn (1962) for pterin analysis.

For the column chromatography, wet-pack unactivated alumina was used. The increasing polarity solvent system outlined by Goodwin (1955) was used. For carotenoid separations, fish were sacrificed by immersion in boiling water. The skin and scales were removed, ground with acetone and extracted with petroleum ether. The acetone dissolved the carotenoids but not the pterins. The petroleum ether solution was then separated on the column. The elutants were analyzed using fluorescent and spectrographic methods.

One- and two-dimensional chromatograms were run on freshly cut fin material. A piece of caudal or anal fin (3mm x 3mm) was cut and squashed on Gelman ITLC Type A unactivated silica gel impregnated sheets. Gelman chromatography chambers and glass "sandwich" chambers were used for development.

For pterins, the solvent systems used were 28% NH_4OH:1-propanol (1:1, 2 hours) and 1-butanol:acetic $acid_{(gl)}$:water (20:3:7, 2½ hours). Each was used separately for 1-dimensional chromatograms (Mertens & Bennett, 1960; Hadorn, 1962). Two-dimensional chromatograms were produced using the method of Hadorn (1962) in which the ammonia-propanol system was run in one direction and, after drying, the butanol-acetic acid-water system run perpendicular to the first producing second and better separations.

Detection methods for these systems included fluorescence, iodine vapor, and $KMnO_4$-acetic acid medium placed on fluorescing spots.

One-dimensional carotenoid chromatograms used a chloroform: ethanol (97:3, ½-1 hour) solvent system with detection methods as listed, but including ordinary light observation.

RESULTS OF PTERIN PIGMENT ANALYSIS

The nitrogenous, fluorescent pterin pigments are separable from carotenoids on the basis of solubility. Both the red and yellow mutants showed good pigment separation with the propanol-ammonia system. The butanol-acetic acid-water system was useful only for 2-dimensional chromatograms.

Poor separations occurred with the Drosophila eyes. The

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reason(s) for this are unknown. One factor may be the wild-type Drosophila used as the standard. A strain known as Swedish-B was used which probably differed from Hadorn's. Since chromatographic information was not available for this strain, Hadorn's published results were used as standard.

Samples of one- and two-dimensional pterin chromatograms are shown in Figures 1-5. We believe red pterins were present in the red mutant but absent in the yellow. The red mutant also produced isoxanthopterin, xanthopterin, 2-amino-4-hydroxypteridine, biopterin, sepiapterin and isosepiapterin. Quantitative increases in isoxantho- and/or xanthopterin occur in the yellows. Yellow mutants also lack 2-amino-4-hydroxypteridine and biopterin. A "modified" blue fluorescing substance (xanthopterin-like pterin?) replaces the red drosopterins in the yellow mutant. These results are summarized in Table 1.

TABLE 1. PTERIN PIGMENTS IN RED AND YELLOW MUTANTS OF *Betta splendens*.

RED	YELLOW
Drosopterins	"Modified" blue fluorescing substance (xanthopterin-like pterin?)
Isoxanthopterin	Isoxanthopterin
Xanthopterin	Xanthopterin
2-Amino-4-hydroxypteridine	—
Biopterin	—
Sepiapterin	Sepiapterin
Isosepiapterin	Isosepiapterin

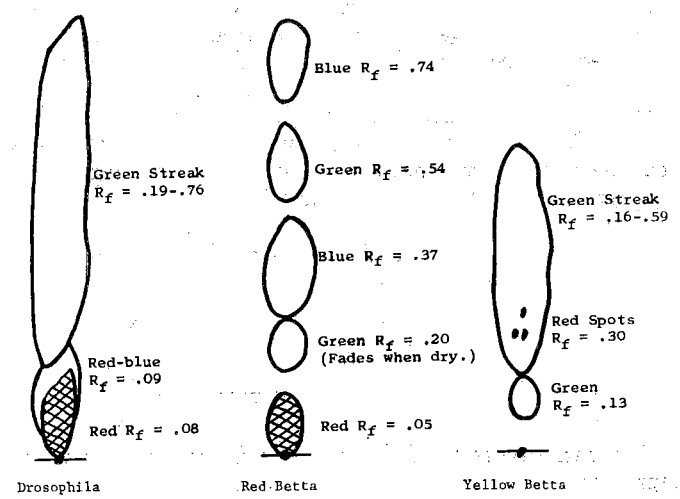


Figure 1. 1-Dimensional pterin chromatograms for 1:1 1-propanol:28% NH_4OH.

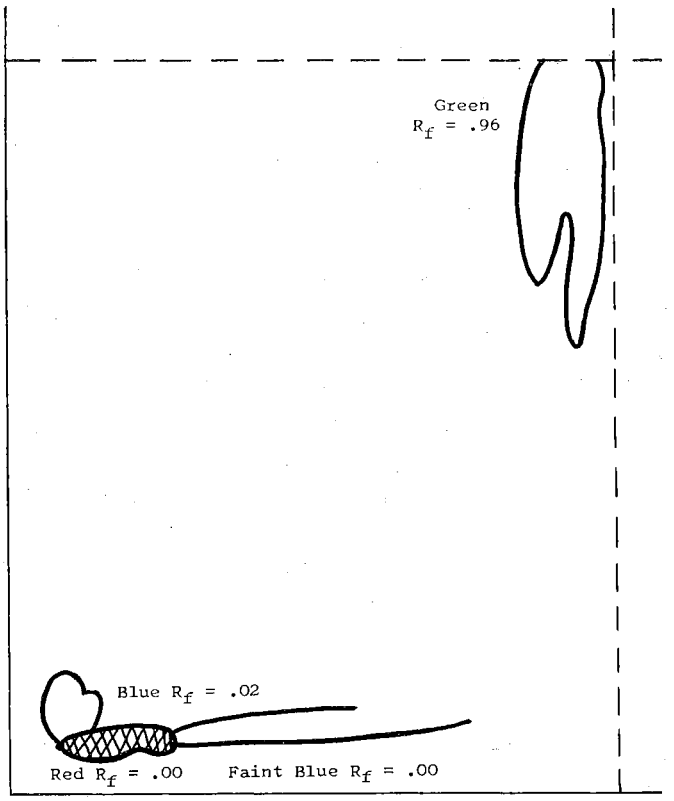


Figure 2. 2-Dimensional pterin chromatogram of *Drosophila melanogaster*.

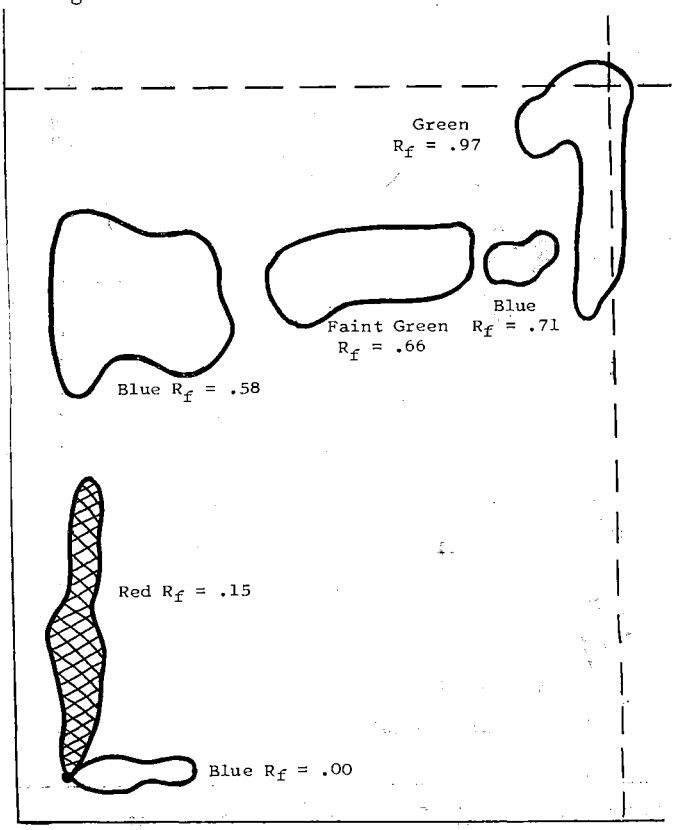


Figure 3. 2-Dimensional pterin chormatogram of red mutant, *Betta splendens*.

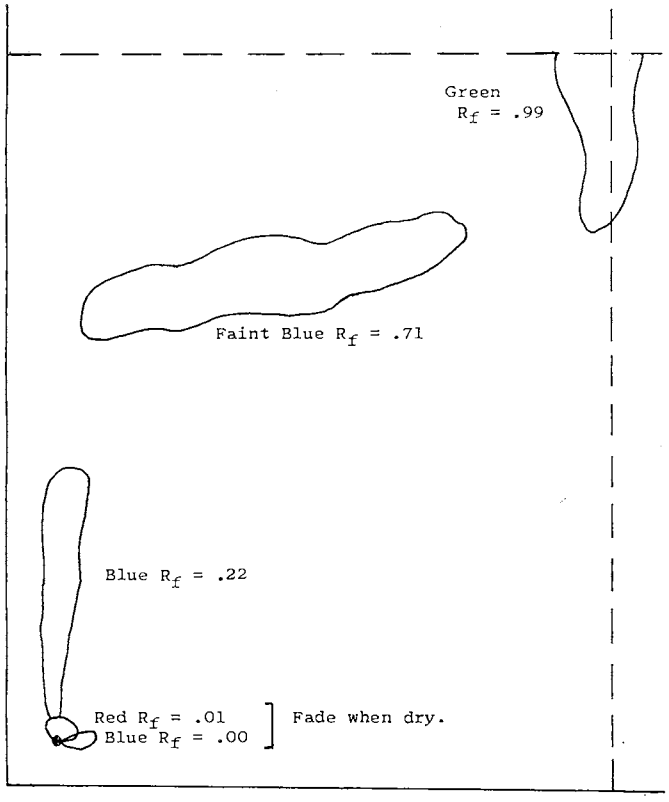


Figure 4. 2-Dimensional pterin chromatogram of yellow mutant, *Betta splendens.*

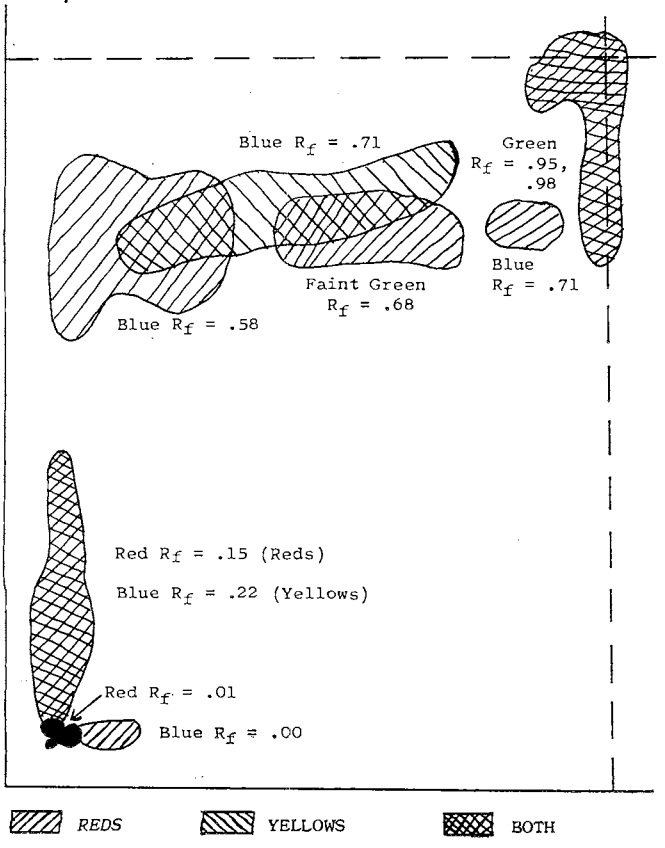


Figure 5. Combined 2-dimensional pterin chromatograms of red and yellow mutants, *Betta splendens.*

RESULTS OF CAROTENOID PIGMENT ANALYSIS

Lipid soluble carotenoids were found in all specimens. The red mutants possess a red-orange carotenoid we believe to be astaxanthin. This was not found in yellows. Lutein, though reported by Goodrich, et al. (1941) was found in neither mutant. Colorless fluorescing carotenoids, possibly phytoene and phytofluene, were found in both.

DISCUSSION

It appears likely that two pigmentary systems produce red Betta color, and that yellow Bettas are mutants unable to produce red. Incomplete synthesis of red pterins (drosopterins) and the red-orange carotenoid (astaxanthin) results in a yellow or colorless phenotype.

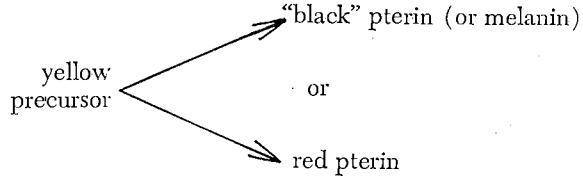
These results do not agree with those reported by Goodrich, et al. (1941). They viewed mutant varieties as expressions of different pigments rather than a block in one type of pigment formation or possibly even a quantitative reduction in one pigment allowing expression of another which was formerly masked.

Results of this experiment indicate both qualitative and quantitative pigment differences for red and yellow Betta mutants. Red drosopterins (= erythropterin? of Goodrich, et al., 1941) and astaxanthin color the red, while both are absent in the yellow.

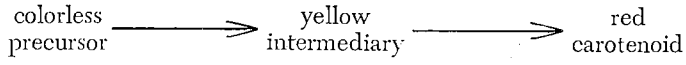
A pattern similar to that of *sepia* Drosophila eye pigments, as reported by Ziegler (1961), emerges for yellow Bettas. Red drosopterins are lacking but increases in isoxantho-, xanthopterin, a xanthopterin-like pterin, and yellow pterin occur. A milder solvent (e.g., propanol:1% ammonia, 70:30) might show only a tetrahydrobiopterin derivative present in the yellow mutant.

In light of genetic information by Lucas (1968) and the results of this experiment, biosynthetic pathways for Betta pterin and carotenoid production can be hypothesized.

Pterins have been shown to be related to melanin with pigments present in the same chromatophores (Fox & Vevers, 1960). A black mutant is probably a phenotypic display of melanin, while red mutants display pterins. Conceivably, both mutants possess a "yellow" precursor which possibly represents an intermediary in melanin and/or pterin synthesis. The melanin-producing enzyme system may be "weakened" because of low riboflavin and high xanthopterin concentrations. Melanogenesis may even stop at a "red" stage. Increased xanthopterin (thought to inhibit melanogenesis) could strengthen the pterin system thus producing "weak" black Bettas that easily undergo color loss, or, if the xanthopterin is "strong enough, melanogenesis could be stopped and red pterins predominate. Selection of the following pathway for pterins best explains all available information:



The pathway selected for carotenoid production may be represented by:



Further research is needed to confirm these pathways and also the relationship, if any, of the carotenoid system to pterinogenesis.

A dual red pigment system indicates four possible phenotypes; "Red" having both red pterins and carotenoids, "Pterin-red" having pterin red and no red carotenoid, "Carotenoid-red" having red carotenoid and no red pterins, and "Non-red" lacking both red pterins and carotenoids.

Genetic information and careful examination of red phenotypes is needed to confirm the presence of two red pigment forming systems in Bettas. Crosses should be made to determine if different types of red can be isolated, if and how they are related, and if and how they might be dependent on other factors.

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