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CAROTENOIDS PRODUCE FLUSH IN THE ELEGANT TERN PLUMAGE[1]

JOCELYN HUDON[2] AND ALAN H. BRUSH[3]
Department of Physiology and Neurobiology, U-42, The University of Connecticut, Storrs, CT 06269

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Adult Elegant Terns (*Sterna elegans*), Roseate Terns (*S. dougallii*), many gulls (Laridae), several pelicans (*Pelecanus* spp.), the Common Merganser (*Mergus merganser*), and ptarmigans (*Lagopus* spp.) display a pink flush on their white body feathers (Stresemann 1927–1934; see also Völker 1964, Höhn and Singer 1980, Harrison 1983). Called variously "flush," "blush," "bloom," "tint," "wash," or "cast," the color is generally confined to the ventral surface. Characteristically, the color is ephemeral, and fades rapidly (within a few weeks to several years) after the death of the bird (Völker 1964). Neither the origin of the coloration nor its chemical nature have been conclusively elucidated (Höhn and Singer 1980). The ephemeral nature of the coloration is particularly challenging.

Stegmann (1956) suggested that the color might originate from a substance produced by the uropygial gland and deposited on the bird's plumage by preening. He proposed that oxidation and breakdown of the substance could account for the rapid color loss (Stegmann 1956). The secretion of the gland in some gulls and the White Pelican (*Pelecanus onocrotalus*) is bright red (Stegmann 1956). However, not all flushed species produce colored uropygial gland secretions (Höhn and Singer 1980), and other causes of the flush are possible. Colored substances are not mentioned in recent reviews of uropygial gland secretions (Jacob 1978, Jacob and Ziswiler 1982). To our knowledge, no feather pigments or environmentally derived substances have been identified as responsible for the pink flush. Also, structural modifications of the flushed feathers have not been demonstrated (Höhn and Singer 1980). Adventitious, yellow colors found in a variety of birds are produced by environmental ferrous oxide adsorbed to the feathers (Berthold 1967).

The availability of colored feathers from an Elegant Tern gave us the opportunity to investigate the factors responsible for the feather flush. We were interested especially in the possibility that it might be a carotenoid that was applied to the feather surface. Carotenoid pigments are widely distributed in birds and are responsible for many of their brightest colors (reviewed in Brush 1981). Adventitious carotenoids would be particularly sensitive to oxidation. However, carotenoid pigments are commonly deposited inside feathers, not on the surface (Lucas and Stettenheim 1972). We found that carotenoids were indeed implicated in the flush, and were deposited inside the feathers.

MATERIAL AND METHODS

An adult Elegant Tern in full molt with a distinct salmon pink flush was collected at the Bolsa Chica Ecological Reserve (Orange County, California) on 15 September 1988. Flushed contour feathers which originated from unspecified areas on the body were plucked and sent to us for analysis. The feathers were tinted uniformly over both vanes and the rachis. Some of the flushed feathers were kept intact for comparison with washed and decolorized feathers.

We first washed the flushed feathers (ca. 1 g) with a soapy solution (ca. 0.1% v/v, Sweet Life lotion detergent) for 24 hr. About 50 ml of this and subsequent solvents were used for the washes. After the soap treatment the feathers were washed repeatedly with distilled water, and once with acetone for several minutes to remove the water (first acetone wash), and air-dried. We also washed the feathers once with petroleum ether for 24 hr. Subsequently, the feathers were soaked in methanol for an additional 24 hr. We again rinsed the feathers once in acetone (second wash) and redried them. The methanol and second acetone washes were combined for analysis. The remaining pigment was

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[2] Current address: Department of Veterinary Anatomy, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada.

[3] To whom the correspondence should be addressed.

extracted in ca. 20 ml of warm, acidified (HCl) pyridine (Hudon et al. 1989) for 2 hr under dim light. At each step we visually compared the feather coloration with the original material. We also estimated the degree of penetration of the various solvents inside feathers by monitoring the speed and extent of color change of blue-colored Blue-and-Yellow Macaw (*Ara ararauna*) wing feathers. The blue is produced structurally and affected by the penetration of solvents into the feathers.

We forced hydrophobic compounds from each unprocessed wash or extract, except the soap wash, once into hexane in a separatory funnel with distilled water. We washed exhaustively the organic epiphases with distilled water, and stored them over anhydrous sodium sulfate under nitrogen in the dark and cold (4°C). The hexane extracts were concentrated under a stream of air.

We recorded absorbance and individual visible spectra of each hexane extract with a Perkin-Elmer model 552 spectrophotometer. We determined pigment concentration in the different extracts from the absorbance at 450 nm, using an extinction coefficient, $E^{1\%}_{1cm}$, of 2,500 (Britton 1985). We also performed preliminary thin-layer chromatography (TLC) on each extract. All extracts contained essentially the same pigments, and were pooled for subsequent pigment identification. We isolated various sets of pigments by preparative TLC on precoated plates of silica gel (Anasil G, Analabs, New Haven, Connecticut). The pigments were resolved with a solvent mixture of hexane : acetone (3:1), eluted from the gel with acetone and transferred back to hexane.

We chromatographed the different isolated fractions on silica gel (Chromagram sheets, Eastman Kodak Co., Rochester, New York) and aluminum oxide IB plates (Baker-Flex, J. T. Baker Chem. Co., Phillipsburg, New Jersey) in the hexane : acetone solvent system. Pigments were identified on the basis of relative mobility on TLC (R_f) and high-performance liquid chromatography (HPLC), color, and comparison with known standards. We determined R_f values for the carotenoid pigments on preparative plates in the hexane : acetone solvent system. Pigments were separated by HPLC with a Zorbax ODS (Dupont Company, Wilmington, Delaware) reversed-phase column (4.6 mm i.d. × 25.0 cm) by elution with an acetonitrile : dichloromethane : methanol (7:2:1) solvent mixture (Nelis and De Leenheer 1983). The pigments were detected at 450 nm. These chromatographic conditions incompletely separated the 3-hydroxy, 4-keto-carotenoids, such as astaxanthin (Hudon et al. 1989). We used standards of canthaxanthin, echinenone, lutein, and zeaxanthin (obtained from Hoffman LaRoche, Basel) for identification. Standard astaxanthin was prepared from lobster (*Homarus americanus*) shell using acidified pyridine. We also used extracts from feathers of the Scarlet Tanager (*Piranga olivacea*), whose pigments have been characterized thoroughly (Brush 1967; Hudon and Brush, unpubl.).

We performed several chemical tests on putative astaxanthin isolated from the feather flush to establish its chemical structure. The tests included the borohydride reduction of carbonyl groups in methanol, the acetylation of hydroxyl groups with acetic anhydride in dry pyridine, and the oxidation of acidogenic carotenoids in alkaline methanol (Hudon et al. 1989).

RESULTS

In total, we obtained about 6.9 μg of pigmented material from sequential washing of 1.13 g of feathers (concentration: 6.1 μg/g of feather, based on the extinction coefficient for carotenoids). About 35% of the total extracted pigments appeared in the first acetone wash. A negligible 5% was extracted with petroleum ether. These treatments only slightly affected the apparent color of the feathers. Subsequent methanol and acetone washes greatly diminished the remaining flush, and the color that remained was largely limited to the central rachis. The combined two latter washes contained over 50% of the extracted colored material. These treatments reduced the intensity of the flush but did not affect its hue, showing that pigments were not extracted differentially. We collected the remaining 10% of colored material through harsher pyridine extraction. After these treatments the feathers were essentially colorless. The feather structure appeared unaffected by the treatments, as revealed under a dissecting microscope.

Initial TLC separated two groups of pigments of different mobility and color. There was a poorly resolved fast yellow pigment (R_f = 0.44–0.77) and a set of five finely resolved, predominantly orange chromophores (R_f's = 0.13–0.31). Because a substance(s) carried from the methanol wash greatly impeded subsequent chromatography, we separated and recovered the two pigment groups on preparative TLC. The refractory material then co-chromatographed with the faster yellow pigments.

Ultraviolet-visible spectra of the fast pigment revealed a featureless, rapid rise in absorbance toward the short wavelengths. The pigment appeared to constitute about a quarter of the total pigmented material. It was not investigated further. The composite spectrum of the predominant set of slower, orange pigments was highly reminiscent of that of a hydroxy-carotenoid (xanthophyll). The visible spectrum exhibited a broad absorption peak at 450 nm, with weak shoulders at 472 nm and 423 nm.

At least five pigments were resolved among the slower group of chromophores. We identified these pigments as: (from fastest to slowest) canthaxanthin (β-carotene-4,4′-dione: R_f = 0.31), phoenicoxanthin (3-hydroxy-β-carotene-4,4′-dione: R_f = 0.23), probable 4′-hydroxy-echinenone (4′-hydroxy-β-carotene-4-one: R_f = 0.20), astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione: R_f = 0.17), and a dihydroxyl xanthophyll (R_f = 0.13). The presence of canthaxanthin and zeaxanthin (β-carotene-3,3′-diol, a xanthophyll) was confirmed on HPLC. No lutein (β,ε-carotene-3,3′-diol) was detected. Presumed phoenicoxanthin and astaxanthin bound tightly at the origin of a plate of aluminum oxide as previously demonstrated for acidogenic carotenoids of 3-hydroxy, 4-keto configuration (Hudon and Brush 1989).

The bulk of the extracted carotenoids was unesterified astaxanthin. By every chemical test (oxidation, reduction, and acetylation), the isolated pigment was identical to an astaxanthin standard. From the spec-

trum (λ_{max} = 466 nm), chromatographic, and chemical evidence we conclude that astaxanthin is the major pigment in the flush of the Elegant Tern.

DISCUSSION

Carotenoids were a prominent pigmentary component in the flushed feathers of the Elegant Tern and in all probability produced the coloration. The perceived color is one expected from the deposition of keto-carotenoids such as astaxanthin. Astaxanthin, the main pigment in the tern, is responsible for the characteristic tissue coloration of many fishes (Simpson et al. 1981). These colors are characteristically "salmon pink." Astaxanthin in feathers is bright red at moderate to high concentration (Brush and Allen 1963). The disappearance of the color from the feathers as pigmented material was extracted further verifies a causal relationship between pigment and coloration.

The small quantity of plumage carotenoids obtained (several micrograms of carotenoids per gram of feathers) can account for the general paleness of the flush. Previous determinations of carotenoid concentration in feathers ranged from a few milligrams of carotenoids to tenths of milligrams per gram of feather (Hudon and Brush 1989, Hudon et al. 1989). Limited observations gave no indications of a structural explanation for the color. Also, feather structure was only minimally disturbed by our manipulations.

Most of the pigments in the Elegant Tern are unlikely to be on the feather surface, where they could easily be oxidized. Petroleum ether, normally a good carotenoid solvent, extracted only about 5% of the pigment from the feathers. But, petroleum ether is a poor extractant because it does not easily penetrate feathers, and enters only feathers that are cut or broken. Solvents with greater penetration, such as acetone and methanol, extracted considerable amounts of pigments from the tern feathers. Additional evidence for internal deposition of the pigments is that still-ensheathed growing feathers were brightly colored. Moreover, the carotenoids were unesterified, unlike skin carotenoids in other birds (Czegzuga 1979). The internal deposition of carotenoids contradicts Stegmann's hypothesis of the application of secreted pigments onto feathers.

The relative ease with which we extracted feather carotenoids with neutral organic solvents provides a possible explanation for the instability of the coloration. Typically, carotenoid-bearing feathers do not yield perceptible amounts of carotenoid pigments to methanol (Völker 1964, pers. observ.). However, Völker (1964) reported easy extraction of carotenoids with the light-sensitive, red feathers of the Crested Quetzal (*Pharomachrus antisianus*). We concur with Völker (1964) that both the ease of pigment extraction in methanol and the light sensitivity of the color could be due to a loose association of the pigments within the feather material. We verified that in typical red feathers (Northern Cardinal, *Cardinalis cardinalis*) the carotenoid pigments are tightly bound to feather proteins (unpubl. observ.). Sensitivity to oxidation and covalent bond breakage could be greatly reduced by a close association of pigments with protein components in these normal feathers. The pigments responsible for the feather flush in the tern presumably are not bound in this manner to feather proteins.

Most birds with a feather flush also display red coloration in the soft integument (legs, bill, combs) and have the capacity to deposit red keto-carotenoids. Among larids, the red, pink, orange, or yellow skin of the legs and feet of several gulls contain carotenoids (Lönnberg 1930, 1934). The available chemical evidence implies these birds can process carotenoids metabolically. Carotenoid pigments are not produced de novo in birds (see Brush 1981) but are obtained initially from the diet. The Elegant Tern's diet consists almost exclusively of fish (e.g., Schaffner 1986). Astaxanthin is found in many fishes and crustaceans (Fox 1976). Astaxanthin in the tern could also be produced enzymatically from dietary carotenoids (see Brush 1981).

Unexpectedly, the brightly colored feathers we examined were formed in the autumn after the terns had ceased breeding. Characteristically, the flush is associated with summer birds (Harrison 1983) and Elegant Terns are reported to lose their flush in the autumn (Monroe 1956). It is generally assumed that the flush is related to breeding, and has a visual function. However, some birds might retain a residual flush in the autumn. The Elegant Tern could acquire a more pronounced coloration in the spring prebreeding molt, which is extensive over the body in the small terns (Cramp 1985).

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