PIGMENTATION IN THE SCARLET TANAGER, *PIRANGA OLIVACEA*

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Conspicuous coloration of passerines and probably of many other birds is important in species recognition and sexual behavior (Hamilton, 1961). The signal functions of plumages have accompanied the evolution of sexual dimorphism and play an important role in the maintenance of the integrity of the species. The tropical avian subfamily Thraupinae (the tanagers) has many brightly colored members. But in most species the sexes are similarly colored, and the majority of the species are nonmigratory and remain paired throughout the year. Sexual dichromatism occurs chiefly in migratory species and in nonmigratory species of which the male is brilliantly colored and which flock during the nonbreeding season (Skutch, 1954). Nuptial color changes are found less frequently and seem to be limited exclusively to migratory species (*e.g., Piranga*). In spite of the extensive documentation of the occurrence of dichromatism in avian plumage, there is a paucity of data on the chemical nature of this phenomenon.

The bright colors characteristic of the plumage of birds offer challenging problems in the fields of pigment chemistry and biochemical evolution and variation. Except for the work of Fox (1962a, b, c; 1965; 1966), on the display and fractionation of carotenoids in the Ciconiiformes and the work of Völker and his colleagues on the canary (1961, 1962), there is relatively little known about the metabolism and biochemistry of avian carotenoid pigments. There have been no extensive investigations of variability in pigmentation on the generic or specific level, and there is only the work of Test (1942) on the pigmentation of hybrid birds. The following report concerns the seasonal and sexual variation of pigmentation in the Scarlet Tanager, *Piranga olivacea*, and includes comparative data from other North American representatives of the genus *Piranga*.

METHODS

Pigments from the feathers and liver of fall and spring specimens of both sexes were extracted in alkaline 95 per cent ethanol. In most cases initial partition resolved the crude extracts into epiphasic components (preferentially soluble in petrol ether) and hypophasic components (preferentially soluble in the aqueous ethanol layer). No interfacial salts were formed during the partitioning procedures, and acidogenic fractions were therefore assumed to be absent. The neutral fractions were washed three times with the opposite solvent and then, when necessary, concentrated by evaporation at room temperature under nitrogen. Pigments prepared by this method did not differ in any way from those prepared by the more complex technique of Blessin (1962), or those extracted with pyridine and then transferred by dilution into *n*-hexane.

Pigments were saponified according to both a modification of the cold method of Booth (1957, p. 59) and by hot saponification with 5 per cent NaOH in 95 per cent ethanol (Fox, 1953; 1962a). There was little or no fatty material present in the feather samples. Fatty material was recovered, however, from the liver extracts. Pigments which remained hypophasic either before or after saponification were forced into petrol ether by the method of Rothblat *et al.* (1963). Occasionally this technique was not completely successful. In these cases the solvent was removed under reduced pressure in a flash evaporator and the pigment redissolved in the appropriate solvent.

Absorption spectra were determined spectrophotometrically on a Cary recording spectrophotometer (Model 11). Partition coefficients were determined by the method of Petracek and Zechmeister (1956). The acid chloroform test for allylic hydroxyl groups (Karrer and Leumann, 1951), reduction of keto groups by sodium borohydride (Krinsky and Goldsmith, 1960), and acetylation with acetic anhydride (Jensen, 1962) were used in the characterization of the pigments.

Pigments were separated by both column and thin-layer chromatography. Column chromatog-

raphy was carried out on both active alumina and MgO:Celite (1:1) under reduced pressure in Zechmeister-Cholonky tubes. A pad of glass wool covered the sintered glass disc at the bottom. Columns were saturated with petrol ether, the column developed with benzene, and the fractions eluted with petrol ether or petrol ether-methanol mixtures. In some cases fractions were removed mechanically, eluted in petrol ether-methanol mixtures and concentrated by evaporation under nitrogen.

Thin-layer chromatography (TLC) was carried out on silica gel plates (Chromatograms, Eastman Co.). Separation was carried out either on the whole plate (20 cm × 20 cm) in the Eastman developing apparatus or on strips (4 cm × 20 cm) in a cylindrical chromatographic tank. A number of solvent systems were tested, but the best results were obtained with benzene:ethyl acetate (2:1) or benzene:methanol (98:2). Extracts of the dandelion and of yellow corn meal were used for comparison. The thin-layer chromatogram provided a simple and rapid method for separation of carotenoid pigments and had a resolving power equal to or greater than the columns used. As many as 10 samples could be conveniently chromatographed simultaneously on a single plate. This was useful in comparative studies.

RESULTS

Spring Males

There was some variability in the absorption maximum of the epiphasic layer produced by partitioning the alkaline ethanolic extract of the contour feathers of spring males. The epiphasic peak in six individuals averaged approximately 460.0 mμ, but the mode was 464.0 mμ. It appeared that this value was crudely correlated with the overall intensity of the plumage. The absorption maximum in hexane was 464.0 mμ. The shape of the curve was characteristic of a keto-carotene (fig. 1a).

Addition of concentrated H_2SO_4 to etheral solutions (or the ethanolic hypophase) produced the blue color characteristic of the polyene pigments (Karrer and Jucker, 1950). The blue coloration was not produced by treatment with concentrated hydrochloric acid, thus eliminating certain classes of carotenoids (aldehydes, epoxides, their furanoid products, and those containing several hydroxyl groups) from consideration.

The partition coefficients at 464.0 mμ were 38:62 in hexane: 95 per cent methanol and 66:34 in hexane: 85 per cent methanol. This corresponds to an M_{50} value of 92 and a relatively polarity of 1.60 (see Krinsky, 1963; Subbarayan *et al.*, 1965, for discussion). Treatment with acid chloroform produced only negligible shifts in the spectra and relative polarity.

The hypophase had an absorption peak in ethanol of 469.5 mμ (fig. 1b). This pigment was forced into petrol ether by the method of Rothblat *et al.* (1964) or by addition of glacial acetic acid. The resultant solutions had absorption peaks at 460.0 mμ (after Rothblat). Column chromatography of the unsaponified hypophase on active alumina produced a major orange band with a single broad peak at 460.0 mμ and a faint, minor band too dilute to recover.

Total extracts of the feathers from spring males in pyridine had a main absorption peak at 483.0 and minor peaks at 465.0 and 427.0 mμ (fig. 1d). The epiphasic pigment obtained by partitioning alkaline ethanol extracts with petrol ether when dried and then redissolved in pyridine gave an absorption curve with a single peak at 486.0 mμ. Thin-layer chromatography resolved the pyridine extract into three components. The mobility of the main fraction (most intense) corresponded to that of the major ambiphasic pigment recovered from ethanolic extracts (see below). The minor fractions in pyridine corresponded in mobility to a minor fraction occasionally observed in TLC separations of the epiphase in hexane.

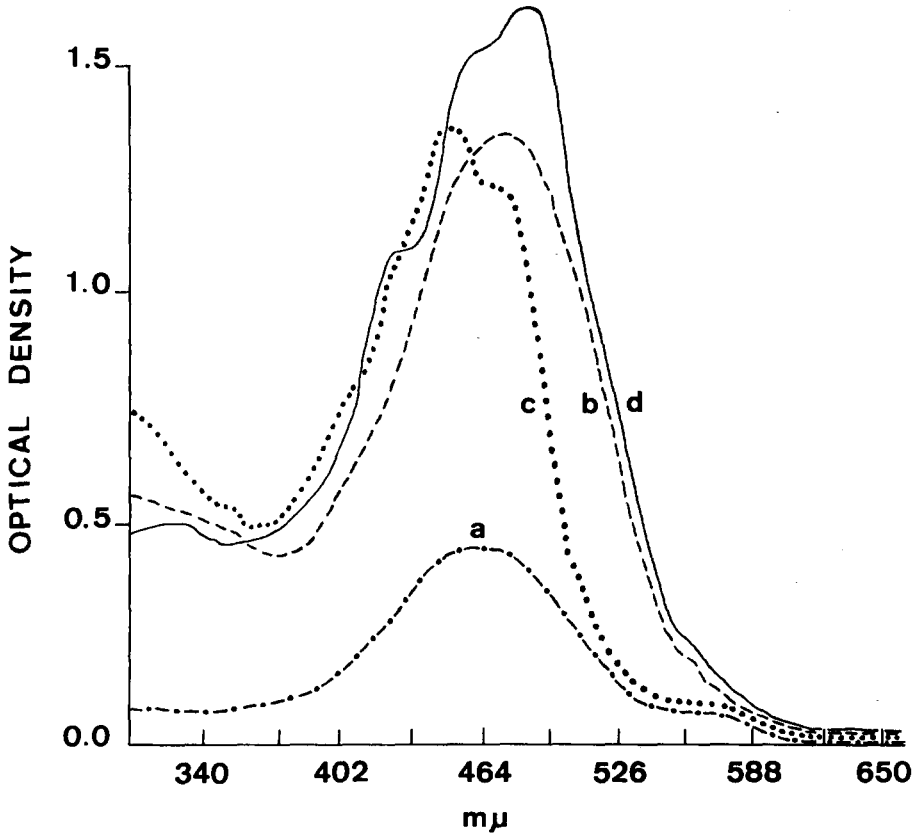

Figure 1. Spectral curves of extract from spring plumage of male *Piranga olivacea*. a = epiphase in petrol ether, λ = 464.0 mμ; b = hypophase in ethanol, λ = 469.5 mμ; c = hypophase after treatment with $NaBH_4$; d = total extract in pyridine; λ = 483.0, 465.0, and 427.0 mμ.

Total pyridine extracts when transferred directly to hexane had an absorption peak at 468.0 mμ. Thin-layer chromatography showed three components, identical to those above, and alumina column separations produced a fast main band (λ = 468.0 mμ) and a single, slower minor band.

Treatment of the pyridine extract with acid anhydride (Jensen, 1962) produced no spectral change. This, plus the negative result of the acid-chloroform test, eliminated the possibility of the presence of allylic hydroxyl groups in this pigment.

Treatment of the hypophase of the feather extract with sodium borohydride in ethanol converted the typical keto-spectra to the hydroxyl form (fig. 1c). The epiphase did not react. The reduced keto compound had a chromatographic mobility and a relative polarity similar to that of the hypophasic pigment from the feathers of fall males (see below). The displacement of the absorption spectrum indicates that the keto group is conjugated with a double ring bond and not with a bond in an acyclic portion of the chromophore.

A residual carotenoid extracted from the black wing feathers had an absorption spectrum which contained elements of both the keto-carotene present in the spring

TABLE 1

EFFECT OF VARIOUS TREATMENTS ON PHASIC PRODUCTS OF ETHANOLIC EXTRACT OF CONTOUR FEATHERS OF MALE SCARLET TANAGERS IN SPRING PLUMAGE

Sample[a]	Treatment	R_f[b]
H, E	None	85–88
E	Saponification	85–88
H	Saponification	70
H	Saponification and partitioning	84; 70

[a] H = hypophase, E = epiphase. The residual pigment extracted from the black wing feathers was strongly hypophasic before and after saponification and had an $R_f = 40$.

[b] Chromatographic solvent = benzene: ethyl acetate 2 : 1.

male and the hydroxy-carotene present in the fall birds (see below). Some of the chemical characteristics were similar to those of the pigment from the contour feathers of spring females. Because only small amounts of pigment were recovered, even under the best possible conditions, the possibility that this pigment is the intermediate mono-keto-mono-hydroxy form could not be eliminated.

Thin-layer chromatography of the epiphase and hypophase obtained by partitioning the unsaponified ethanolic extract of contour feathers produced a single fraction with an R_f of 85–88 (table 1). After saponification the spectra remained unchanged (total extract (in ethanol) = 469.5 mμ; epiphase = 464.0 mμ; hypophase = 464.0 mμ). This result, again, suggests slight spectral shifts due to concentration changes. There was no change in the mobility of the epiphasic fraction, even after such severe treatment as heating to dryness in air and redissolving. Saponification of the hypophase (by heating with 5% NaOH) reduced the R_f to 69.6. Although there was little free fat, a greasy spot remained at the application point on the chromatographic plate. Subsequent partitioning of this solution produced a hypophase with an $R_f = 70$ and an epiphase with an $R_f = 84$. Thus the mobility of the hypophase was affected by saponification, without any spectral changes.

These data suggest that there may be but a single ambiphasic pigment present in the feathers of mature, spring male individuals. Its spectral characteristics and partition behavior suggest that the pigment is canthaxanthin or 4-4′-diketo-β-carotene. Fox and Hopkins (1965) reported a similar pigment in the feathers of several Ciconiiformes. A second pigment with a similar spectrum but slightly different partitional and chromatographic behavior may be present but is revealed only after saponification. This may be the intermediate compound in the conversion of the yellow pigment of fall males to the red of the spring birds. It was present in extremely small amounts in the contour feathers, and attempts at isolation were unsuccessful.

The main pigment extracted from the spring plumage of male Summer Tanagers (*P. rubra*), Hepatic Tanagers (*P. flava*), and the head of the Western Tanagers (*P. ludoviciana*) has spectral, chemical, and chromatographic properties identical to the pigment in *P. olivacea*. On the basis of this evidence I conclude that it is also canthaxanthin.

SPRING FEMALES

Pigments extracted from the feathers of spring females behaved quite differently from those recovered from males. Upon partitioning between petrol ether and 95

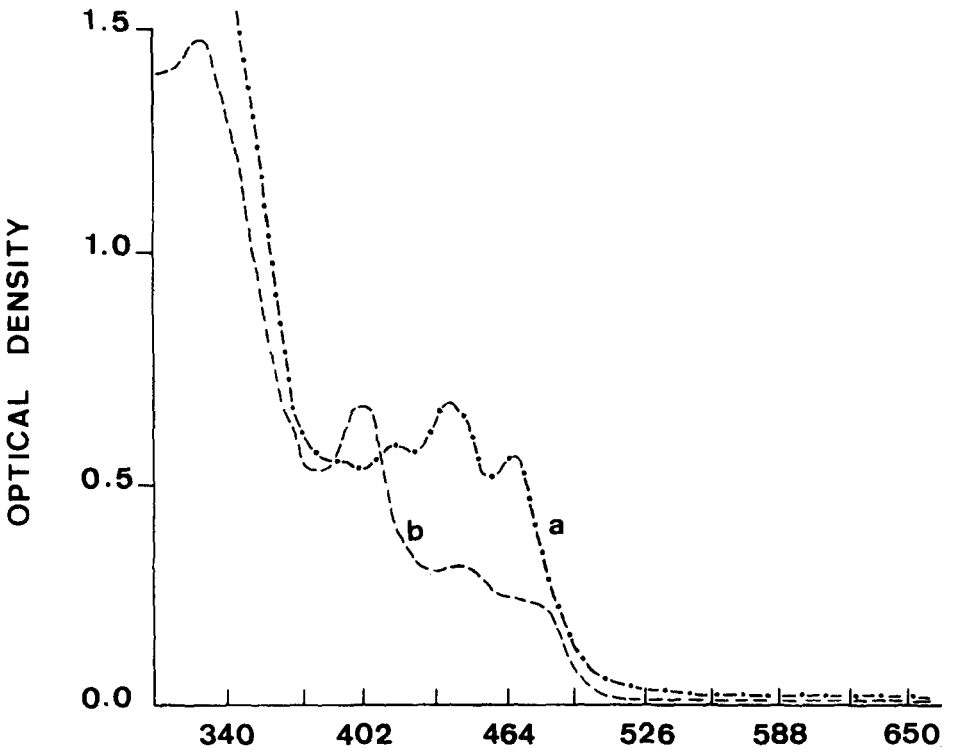

Figure 2. Spectrum of typical hydroxy-xanthophyll pigment extracted from liver and feathers of fall *Piranga olivacea* of both sexes. Saponified liver or feather pigments (curve a) have curves with peaks at 469.5, 438.5, and a shoulder at 410 mμ (solvent = petrol ether or pyridine). Crude ethanolic hypophase pigments have a major peak near 400 mμ, with minor peaks at higher wavelengths (curve b).

per cent ethanol, the pigment remained entirely hypophasic. The pigment could not be forced into petrol ether by the Rothblat technique. After cold saponification the spectrum in petrol ether was extremely poor and had a single broad peak at about 450 mμ. This solution showed a single yellow spot on TLC with an R_f of 76.7. Hot saponification was no more successful. Flash evaporation (from alkaline or neutral ethanol) produced an amorphous solid, which was insoluble in most common organic solvents. The spectrum in methanol-benzene was a simple hyperbolic curve.

Fall Males

The hypophase recovered from the feathers of fall males had a single broad absorption peak at 468.5 mμ and produced a single chromatographic fraction with an R_f of 74. This pigment had a partition coefficient of 93:7 (85% ethanol: petrol ether). The epiphase had a spectrum in petrol ether with peaks at 468.5 and 438.4 mμ and a broad shoulder at 410 mμ. Thin-layer chromatography resolved the epiphase into two fractions with respective R_f values of 74 and 65. Partition coefficients of the epiphase gave an M_{50} of 87.5 and a relative polarity of 1.70–1.75. The spectrum of this pigment was typical of xanthophyll carotenes, and its chemical

behavior identified it as a dihydroxy carotene, probably isozeaxanthin, 4-4′-dihydroxy-β-carotene.

The crude ethanolic extract from the liver of the fall male Scarlet Tanager had a major peak at 396.5 mμ, and minor, rather broad peaks at 433.0 and 474.0 mμ (fig. 2b). Both the hypophasic and epiphasic fractions had spectra with peaks at 471.2, 443.8, and 412.9 mμ (fig. 2a). Both fractions were resolved chromatographically into two fractions with respective R_f values of 78 and 54.

Fall Females

The epiphasic feather pigment of fall females was identical to that in fall males. The absorption spectrum had peaks at 469.5, 438.5, and a shoulder at 410 mμ. The single pigment had an R_f on thin-layer chromatography of 73.5 but lacked the slower component present in the male. When transferred into petrol ether, the hypophasic pigment behaved chromatographically as the epiphase.

As indicated previously, crude ethanolic extracts of the liver showed identical spectra in both sexes. Epiphasic and hypophasic fractions recovered from partitioning with petrol ether had peaks at 469.5, 438.0, and a shoulder at 410 mμ. Thin-layer chromatography of the unsaponified components produced two fractions with R_f values of 41 and 26, which presumably represent the xanthophyll and its ester. This mobility was different from that of the pigments recovered from the livers of fall males (R_f = 78 and 54).

Total pyridine extracts of the feathers and liver from fall females gave a yellow solution with the typical hydroxy-xanthophyll spectrum. Treatment with acid anhydride produced spectral shifts indicative of the presence of allylic hydroxyl groups. Acetylation also reduced the chromatographic mobility of this pigment.

After saponification of the crude ethanolic extract, the mixture from female livers was partitioned and the hypophase forced into petrol ether. The hypophase in petrol ether had a spectrum with peaks at 468.5, 439.4, and 412.9 mμ (fig. 2a). This pigment had a partition coefficient which had an M_{50} of 85 and a relative polarity of 1.75.

Epiphasic fractions from both saponified and unsaponified ethanol extracts had absorption peaks identical to those in figure 2 (peaks at 468.5, 439.4, and 412.9 mμ). Hypophasic components after similar treatment gave the same spectra.

Saponification of the liver material affected both its mobility and the number of fractions present (table 2). Unsaponified hypophasic material forced into petrol ether gave two fractions in TLC with low mobilities (R_f = 35). If the extract was partitioned, and then the hypophase saponified, two fractions were present, and they had increased mobilities (R_f = 58.5 and 41.0) when compared with the unsaponified material. The epiphase in the latter case produced a single fraction with a mobility (R_f = 57.5) similar to the more mobile hypophase fraction but somewhat different from that of the feather epiphase.

The pigments of the female, although spectrally identical to those of the fall male, are more strongly hypophasic. This difference reaches its extreme in the hypophasic behavior of the feather pigment of spring females. The data from saponification and partitioning experiments (table 2) suggest that the pigment in spring females may exist as a free xanthophyll, whereas in the fall female it is partly esterified and the pigment in the fall male mostly esterified or conjugated to some other class of compound.

TABLE 2

EFFECT OF VARIOUS TREATMENTS ON THE CHROMATOGRAPHIC BEHAVIOR OF PHASIC PRODUCTS OF LIVER CAROTENOIDS FROM FALL SCARLET TANAGERS[a]

Treatment	R_f	
1. Ethanolic extract saponified, partitioned with petrol ether		
Hypophase forced into petrol ether		35.0
2. Unsaponified ethanolic extract partitioned with petrol ether		
Epiphase	57.5	—
Hypophase, unsaponified, forced into petrol ether	41.0	26.0
Hypophase, saponified, forced into petrol ether	58.5	41.0
3. Male, ethanol, extract, unsaponified		
Epiphase, hypophase forced into petrol ether	78.0	54.0

[a] Solvent = benzene: ethyl acetate 2 : 1.

Carotenoids similar to those described for the plumage of fall female Scarlet Tanagers were also found in the females of the Western Tanager, *P. ludoviciana*, the female Summer Tanager, *P. rubra*, and the body (but not the head) contour feathers of the male Western Tanager.

DISCUSSION

The seasonal change in the plumage color in the male Scarlet Tanager appears to be due to the oxidation and subsequent reduction of a xanthophyll carotenoid. The spectral, partitioning, and chemical data suggest that the major pigment in spring males is probably 4-4′-diketo-β-carotene. This pigment, canthaxanthin, is also present in the body plumages of male Summer and Hepatic Tanagers and the head of the Western Tanager. Canthaxanthin has also been reported from the tissues of a variety of Ciconiiformes (Fox, 1962a, b, c). The pigment in the fall male Scarlet Tanager and the females (both spring and fall plumages) of all four species appears to be isozeaxanthin (4-4′-dihydroxy-β-carotene). Although little is known about the exact nature of the interconversion of the two pigment forms, one may presume that they have common precursors, *e.g.*, carotenoids derived from the insects and fruits which form the diet of these birds. The conversion of the pigment (a dehydrogenation), which accompanies the prenuptial molt, is presumably mediated by a single enzyme, but may occur in two steps. The site of this event is not known, but similar pigments occur simultaneously in the feathers and the liver. The scanty evidence now available suggests that an intermediate compound, 4-hydroxy-4′-keto-β-carotene, may be found in the melanistic remiges and rectrices of the male Scarlet Tanager.

The variation in the wavelength of the spectral maximum of the feathers of spring male Scarlet Tanagers may reflect the variation in plumages in populations and even in feathers of individuals. Occasionally, individual birds had considerable numbers of yellow feathers intermixed with the red. This was especially true during periods of molt. Such a situation leads to ambiguities in the description of these plumages and to problems in establishing precisely the spectral maxima of such mixtures. As has been pointed out previously (Fox, 1962; Brush and Reisman, 1965), pigment concentration also may affect spectral behavior. This condition was present in the feathers of all male tanagers. The feather pigment in more intensely pigmented individuals tended to show slight spectral shifts to longer wavelengths

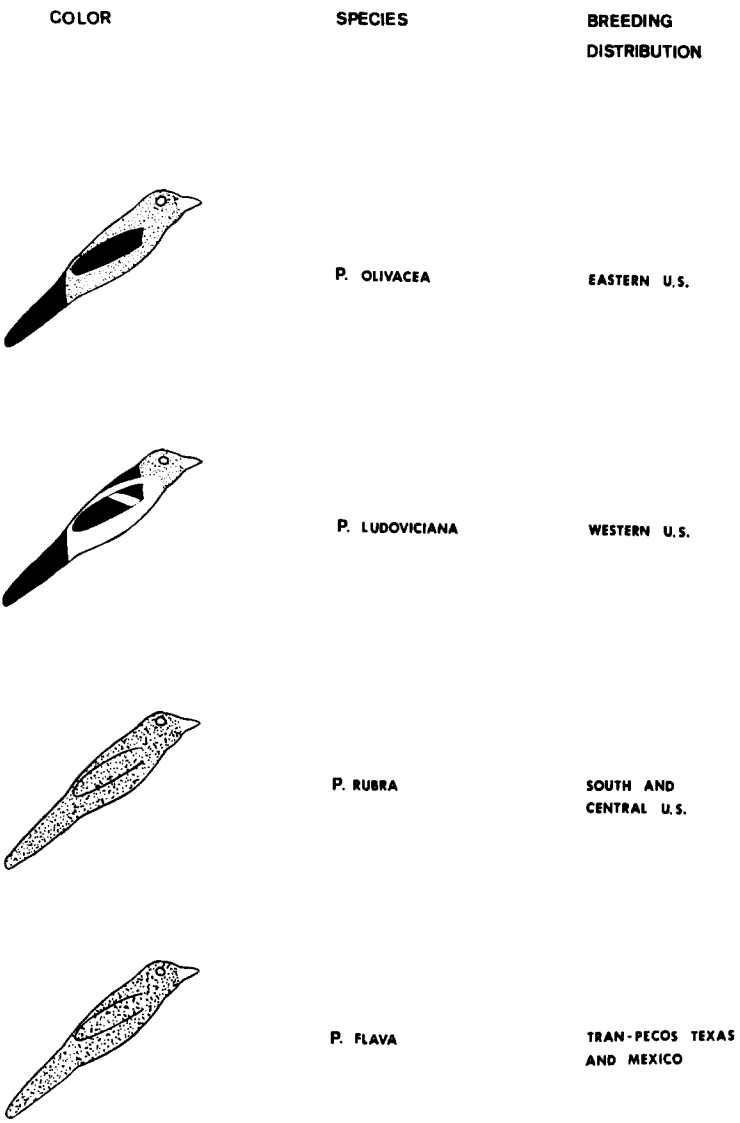

Figure 3. Color patterns (stippled = red, blank = yellow) and breeding distribution of North American tanagers. Patterns represent plumage of adult males in spring. See text for discussion.

when compared with the less intensely pigmented individuals. However, this was apparently not the case in birds in fall plumage. Although Roberts (1955) and Dwight (1900) have claimed that color intensity was a useful characteristic for distinguishing sexes in fall plumage, there were no spectral differences between the more intensely colored yellow males and the duller females used in the present study. There were chromatographic differences between the pigments in the liver, but not the feathers, of the sexes in the fall. The significance of this difference is not known.

The seasonal change in the plumage coloration of tanagers undoubtedly is under hormonal control, although the actual conversion of pigments probably is mediated by an enzyme. The relation between this change and the mobility differences observed between the pigments of the sexes is still unclear. It is interesting to note that the spring molt of male Scarlet Tanagers is incomplete. Only the brightly colored, carotenoid-pigmented, contour feathers are replaced. On the other hand, the postnuptial (prebasic) molt is complete. The residual carotenoid pigment found in the highly melanistic wing and tail feathers may be either a mixture of keto- and hydroxy-carotenes or a metabolic intermediate.

The four species of the genus *Piranga* present as breeding birds in North America presumably evolved from a Central American stock of brightly, but uniformly, colored ancestors (fig. 3). Judging by the current geographic distribution of breeding areas and by feather color and pattern, the ancestral form may have been similar to the Hepatic Tanager, *P. flava*. The male Hepatic Tanager is uniformly red in color. Its range includes Central and South America, but it reaches northward into the extreme southwestern United States. A superficially similar form, the Summer Tanager, *P. rubra*, has a similar color pattern but a broader breeding range in temperate North America. It is interesting to note that the adult male plumage shows no seasonal color variation, even though there is a complete postnuptial molt. Therefore, sexual dichromatism in *P. flava* and *P. rubra* is maintained all year. The Scarlet Tanager, as described in this report, has red contour feathers and black remiges and rectrices and is only seasonally dichromatic. The male Western Tanager, *P. ludoviciana*, is characterized by a red head, yellow body, and black rectrices and remiges. The pigment in the body contour feathers is that of the female and fall males. The pigment in the feathers of the head is identical to that of the spring males. *P. ludoviciana* is also the only species of the group with white wing bars. The pigments and feather patterns are essentially identical in the females of these species. The evolutionary significance of such sexual dimorphism and its role in species formation has been discussed by Sibley (1957).

It is not surprising that there is probably little genetic information contained in the carotenoid pigments of these birds. Carotenoids are not primary gene products but belong to the category of "asemantic molecules" as proposed by Zuckerkandl and Pauling (1965). Such molecules are not synthesized by the organism and therefore do not express, either directly or indirectly, any of the information that the organism contains. Thus carotenoids do not represent a transcription of the genetic material as a protein might. Although vertebrates do not synthesize carotenoids *de novo*, they can be modified extensively once they are assimilated. The plumage patterns that are produced by selective deposition of pigments are certainly under genetic control and have been produced as the result of strong selection pressures on visual communication systems. Such systems are intimately linked to various aspects of the biology of these species which include migration, maintenance of well-defined, strongly defended intraspecific territories, and a short, intense breeding season.

I would suggest, then, that a form similar in color, pattern, and distribution to the Hepatic Tanager gave rise to the three other forms. The genetic information required to produce the resultant color patterns is probably not great, and it appears that selection has favored changes in patterns rather than in the nature of the molecule used to produce these patterns. The three plumage patterns (fig. 3) in this group may have evolved historically in a manner similar to that proposed for species formation in the Parulidae by Mengel (1964). That the signal system

based on the colors and patterns described here is still imperfect is reflected in the occurrence of interspecific hybrids (Cockrum, 1952; Mengel, 1963).

SUMMARY

The seasonal differences in the pigment in the plumages of the male Scarlet Tanager, *Piranga olivacea*, are due to an oxidation and reduction of an oxygen-containing side chain on the cyclic part of a carotenoid pigment. The carotenoid pigment of the fall male is the same as that found in the female in both breeding and nonbreeding plumages. There are differences, however, in the molecules to which the carotenoid is esterified. The pattern of the plumage of *P. olivacea* is compared with other North American species of the genus *Piranga*.

ACKNOWLEDGMENTS

This research was supported by grants from the Office of Research Development, University of Connecticut Research Foundation, and the National Science Foundation (GB-4710). I am grateful to the following individuals who supplied materials used in this investigation: D. Amadon, American Museum of Natural History; E. C. Blake, Chicago Natural History Museum; F. McKenna, Minnesota Natural History Museum; G. Morton, Cornell University; S. Russell, University of Arizona; and L. L. Short, Jr., U.S. National Museum. D. L. Fox, University of California, La Jolla, and P. Stettenheim, Michigan State University, both read the manuscript in early stages and contributed many useful suggestions.

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