tion of this extract indicated that the inhibitory factor is a dialyzable material of low molecular weight. Elution pattern on Sephadex column and electrophoretic studies strongly suggest that it is a protein of low molecular weight. This protein possessing the inhibitory capacity is present in both Opaque-2 and Deccan variety. However, its concentration in Opaque-2 variety is markedly high.

Opaque-2 is known to contain markedly higher water-soluble and NaCl-soluble nitrogen and lower prolamine fraction than normal variety of maize (Jimenez, 1966). Striking differences in the protein fractions have also been observed in developing endosperm of the two varieties (Murphy and Dalby, 1971). According to these workers, the striking differences in the NaCl-soluble nitrogen in Opaque-2 and normal variety were largely accountable by a dialyzable nitrogeneous constituent of low molecular weight. This is in line with the observation of the present investigation on the presence of a protein probably of identical nature but in addition possessing a powerful inhibitory action in relation to aflatoxin production.

ACKNOWLEDGMENT

The authors express their sincere thanks to Coluchur Gopalan, National Institute of Nutrition, Hyderabad, for his keen interest in this investigation. Thanks are also due to P. G. Tulpule and D. N. Roy for their helpful suggestions.

LITERATURE CITED

Adye, J. C., Mateles, R. I., *Biochem. Biophys. Acta* **86,** 418 (1964).
Anandam, R. J., M. Sc. (Ag) Thesis, Andhra Pradesh Agric. Univ., Hyderabad (1970).
Davis, B. J., *Ann. N.Y. Acad. Sci.* **121,** 404, (1964).
Diener, U. L., Davis, N. D., in "Aflatoxin, Scientific Background, Control and Implications," Goldblatt, L. A., Ed., Academic Press, N.Y., London, 1969, p 13.
Doupnik, B., Jr., *Phytopathology* **59,** 1554 (1969).
Goldblatt, L. A., in "Aflatoxin, Scientific Background, Control and Implications," Goldblatt, L. A., Ed., Academic Press, N.Y., London, 1969, p 8.
Hesseltine, C. W., Shotwell, O. L., Ellis, J. J., *Bacteriol. Rev.* **30,** 795 (1966).
Jimenez, J. R., in "Proc. High lysine corn conference," Corn Ind. Res. Foundation, Washington, 74 (1966).
Kriz, H., *Acta Vet. Brno.* **39,** 171 (1970).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 265 (1951).
Murphy, J. J., Dalby, A., *Cereal. Chem.* **48,** 336 (1971).
Osborne, T. B., Mendel, L. B., *J. Biol. Chem.* **18,** 1 (1914).
Rao, K. S., Tulpule, P. G., *Nature (London)* **214,** 738 (1967).
Smith, I., *Chromatogr. Electrophor. Tech. II* 170 (1960).
Tropical Products Institute, Recommended Procedures for Detection and Estimation of Aflatoxin $B_1$ in Groundnuts, T.P.I. Rep. No. G 13, (1965).

Vellore Nagarajan*
Ramesh Venkataramana Bhat

National Institute of Nutrition
Indian Council of Medical Research
Hyderabad-7, India

*Received for review December 8, 1971. Accepted March 7, 1972.*

# Identification of Carotenoid Constituents in *Hibiscus syriacus* L.

The ten carotenoid pigments found in the bud, leaf, and flower of *Hibiscus syriacus* L. were each identified and quantitated. Those most abundant in the buds and leaves were β-carotene and lutein; lutein 5,6-epoxide predominated in the flower. Carotene hydrocarbons comprised 19% of the total carotenoids in the flowers, with 37% in the buds and 33% in the leaves. *Hibiscus syriacus* contained a much lower percentage of the colorless phytoene precursors to the carotenoids than cotton (*Gossypium hirsutum* L.). However, three carotenoid pigments, cryptoxanthin, chrysanthemaxanthin, and antheraxanthin, were present in *H. syriacus* but not in cotton, and two, flavoxanthin and violaxanthin, were found in cotton but not in *H. syriacus*.

Until the discovery by Coad (1914) of the malvaceous plant *Hibiscus syriacus* L. (Rose of Sharon) on which the boll weevil (*Anthonomus grandis* Boheman) could feed, oviposit, and develop, this insect was considered monophagous on cotton (*Gossypium hirsutum* L.)

Until recently there has been general disagreement regarding the classification of the *Hibiscus* and *Gossypium* genera within the Malvaceae family. Fryxell (1968) redefined the tribe Gossypieae (Malvaceae), which includes the genus *Gossypium* (cotton), and specifically separated it from the malvaceous tribe Hibisceae, to which the genus *Hibiscus* belongs. His separation is based on the presence of pigment glands which are associated with the triterpene pigment gossypol (the Gossypieae appear to be unique in possessing these glands).

Recent developments in research on the boll weevil indicate that eradication of this insect may become feasible, but before a wide scale control or eradication program can be initiated, it is essential to determine whether alternate host plants would serve as an endemic source for reinfestation of cotton. Rose of Sharon, because it is used as an ornamental on many farms and is abundant throughout the South and Southeast, is the most important of the alternate hosts. Methods have since been developed for a chemical evaluation of the plant components affecting insect response to the two species and the taxonomic relationship between the species. Thompson *et al.* (1968) identified the carotenoids in buds, leaves, flowers, and other tissues of the cotton plant, and we now report a similar study of the carotenoids of *H. syriacus*. Also, Rose of Sharon is grown as a significant commercial ornamental in the southeastern United States. Since the desirability of varieties is influenced by bloom color, an assessment by plant breeders of the extent to which the carotenoids modify the anthocyanin color could provide background information for the development of new varieties.

## MATERIALS AND METHODS

**Plant Material.** Flowers, leaves, and bracted buds of *H. syriacus* were harvested from mature plants grown in field plots at Mississippi State University.

**Pigment Extraction.** The plant material was macerated in an electric blender with acetone, the acetone was removed

Table I. Concentration of Carotenoids in Plant Parts of *Hibiscus syriacus*[a]

| Plant tissue | Carotenoids mg/kg fresh wt | Ratio of hydrocarbon to oxygenates |
|---|---|---|
| Flowers | 0.8 | 19:81 |
| Buds | 9.7 | 37:63 |
| Leaves | 112.9 | 33:67 |

[a] Quantitative estimation of total carotenoids calculated by using $E_{1\,cm}^{1\%}$ of 2505 for β-carotene at $\lambda_{max}$.

by filtration, and the residue was reextracted until all the pigment was removed. With four extractions, 99.9% of the carotenoids were removed, confirmed by visible spectra of a fifth extraction. The successive acetone extracts were combined, and an equal amount of water was added. The aqueous acetone was then treated with ether (peroxide-free) until two layers formed. The ether layer was dried over anhydrous sodium sulfate, and the solvent was evaporated *in vacuo* at 40°C. Saponification of the residue was accomplished by the method of Pattee and Purcell (1967).

**Chromatography.** Column chromatographic separation of the total pigment into hydrocarbon and xanthophyll fractions was carried out on 5% aqueous deactivated alumina (Baker) with increasing amounts of acetone in petroleum ether (bp 38–60°C). The carotene hydrocarbons were separated by column chromatography on magnesium oxide:Celite (1:1 w/w). The column was developed with increasing amounts of acetone in hexane (Pattee and Purcell, 1967).

The hydroxy and epoxy carotenoids were separated by thin-layer chromatography (tlc) with vegetable oil-coated Kieselguhr (Egger, 1962) in methanol–acetone–water (80:16:12). The tlc plates were prepared with Brinkmann apparatus on 20 × 20-cm glass, 250-μ bed depth, and developed in ascending fashion in solvent chambers saturated with the vapor of the solvent to a height of 10 cm from the start.

**Identification.** The individual pigments were identified by comparing the chromatographic movements and visible spectra with those of authentic samples. Spectral data were obtained with a Beckman DK-2A ratio recording spectrophotometer in 1.0-cm matched cells. Partition coefficients were obtained by the method of Subbarayan *et al.* (1965). Also, the effects of hydrochloric acid in aqueous methanol on the absorption spectra were studied by the method of Curl and Bailey (1954).

The following authentic samples were available for direct comparison: lutein (3,3′-dihydroxy-α-carotene), and auroxanthin (3,3′-dihydroxy-5,8,5′,8′-diepoxy-β-carotene) from the flower of yellow pansy (*Viola tricolor* L.) (Karrer and Rutschmann, 1944); α-carotene (Mackinney, 1935); phytoene (7,8,11,12,7′,8′,11′,12′-octahydrolycopene) (Porter and Zscheile, 1946); phytofluene (7,8,11,12,7′,8′-hexahydrolycopene) (Zechmeister and Sandoval, 1946); lutein 5,6-epoxide (Goodwin, 1955); and synthetic β-carotene obtained commercially.

## RESULTS AND DISCUSSION

**Quantitative Estimation.** The quantitative estimation of the total carotenoids in the various plant parts of *H. syriacus* after saponification are given in Table I in milligram per kilogram of fresh weight calculated by using $E_{1cm}^{1\%}$ of 2505 for β-carotene.

**Carotenoids of Fresh Plant Tissue of *H. syriacus*.** The hydrocarbons of fractions I and II obtained from the alumina column (Table II) were separated by rechromatography on MgO–Celite (1:1 w/w) with acetone in hexane (1, 2, 4, and 5%); β-carotene and phytoene were the principal constituents of the hydrocarbon fraction of the buds; α- and β-carotene predominated in the hydrocarbon fraction of the leaves; smaller amounts of β-carotene and no α-carotene were found in the flower. Much smaller amounts of phytoene and phytofluene were found in the green tissues of *H. syriacus* than in similar tissue from cotton (Thompson *et al.*, 1968). The percentage of β-carotene was more than twice as great in the leaf of *H. syriacus* (56.0) as in cotton (21.2%), and smaller amounts were present in the buds (*H. syriacus* 3.0; cotton 25.1%) and flowers (*H. syriacus* trace; cotton 4.3%).

Fractions III to VI were separated into their components by reverse phase tlc on vegetable oil-coated Kieselguhr in methanol–acetone–water (80:16:12) (Egger, 1962). The 5,6-epoxides and diepoxides were eluted from the alumina column ahead of their corresponding 5,8-isomers. Lutein 5,6-epoxide was converted into flavoxanthin (3,3′-dihydroxy-5,8-epoxy-α-carotene) and neoxanthin (3,3′,5′-trihydroxy-6′-hydro-5,6-epoxy-β-carotene) into neochrome (3,3′,5′-trihydroxy-6′-hydro-5,8-epoxy-β-carotene) by adding a trace of alcoholic hydrochloric acid. The pigment then tentatively

Table II. Carotenoids Obtained from the Fresh Green Tissue of *Hibiscus syriacus*

| Fraction no.[a] | Absorption maxima, nm in hexane | Identity | $E_{1\,cm}^{1\%}$ | $\lambda_{Max}$ | Solvent | Partition coefficient in 75% MeOH–hexane | Total carotenoids, % Buds | Leaf | Flower |
|---|---|---|---|---|---|---|---|---|---|
| I | (299), 285, 271 | Phytoene | 1250 | 285 | Pet ether | ... | 2.7 | Trace | Trace |
| | 332, 347, 364 | Phytofluene | 1350 | 347 | Pet ether | ... | 1.0 | Trace | Trace |
| II | 418, 446, 471 | α-Carotene | 2850 | 446 | Hexane | 0:100[b] | 0.6 | 0.3 | ... |
| | (421), 448, 481 | β-Carotene | 2505 | 448 | Hexane | 0:100[b] | 3.0 | 60.0 | Trace |
| III | 420, 444, 476 | Lutein | 2160 | 444 | Ethanol | 18:82 | 91.2 | 43.5 | ... |
| | 445, 474 | Lutein 5,6-epoxide | 2148 | 445 | Hexane | 0:100[b] | ... | ... | 53.7 |
| IV | 422, 449, 485 | Cryptoxanthin[c] | 2505[d] | 449 | Ethanol | 19:81[b] | ... | ... | Trace |
| | 424, 450 | Chrysanthemaxanthin[c] | 2505[d] | 423 | Ethanol | 26:74 | ... | 0.1 | 10.5 |
| | 421, 435, 444 | Antheraxanthin | 2505[d] | 447 | Ethanol | 34:66 | ... | 0.1 | ... |
| V | 380, 405, 427 | Auroxanthin | 1850 | 405 | Ethanol | ... | ... | ... | 23.1 |
| | 335, 420, 438, 467 | Neoxanthin[c] | 2270 | 438 | Ethanol | 100:0 | ... | Trace | .. |
| VI | 317, 399, 422, 449 | Neochrome[c] | 2505[d] | 422 | Ethanol | 81:19 | 1.5 | ... | 12.6 |

[a] Total pigment chromatographed on 5% aqueous deactivated alumina with increasing amounts of acetone in petroleum ether (bp 30–60°C). [b] Partition coefficient in 95% methanol–hexane. [c] Tentative identification made by comparing the physical and chemical properties with those in the literature. [d] Quantitative estimation calculated on basis of $E_{1\,cm}^{1\%}$ of 2505 for β-carotene.

identified as neoxanthin had spectral and partition coefficient properties corresponding to neochrome (Curl, 1964). Fraction VI contained a single pigment that was tentatively identified as neochrome, since it has spectral properties and a partition coefficient consistent with the values cited in the literature (Subbarayan *et al.*, 1965; Curl, 1964). Fraction IV contained three carotenoids tentatively identified by comparison with the spectral properties and partition coefficients cited in the literature (Jungalwala and Cama, 1962) as cryptoxanthin (3-hydroxy-$\beta$-carotene), chrysanthemaxanthin (3,3′-dihydroxy-5,8-epoxy-$\alpha$-carotene), and antheraxanthin, (3,3′-dihydroxy-5,6-epoxy-$\beta$-carotene), none of which were found in the plant parts of cotton (Thompson *et al.*, 1968). Flavoxanthin (stereoisomer of chrysanthemaxanthin) and violaxanthin (3,3′-dihydroxy-5,6,5′,6′-diepoxy-$\beta$-carotene) were found in cotton plant tissue but were absent in the plant tissue of *H. syriacus*.

ACKNOWLEDGMENT

The authors wish to thank Keeston Lowery of this laboratory for helpful technical assistance.

LITERATURE CITED

Coad, B. R., *J. Agr. Res.* **2,** 235 (1914).
Curl, A. L., J. AGR. FOOD CHEM. **12,** 522 (1964).
Curl, A. L., Bailey, G. F., J. AGR. FOOD CHEM. **2,** 685 (1954).
Egger, K., *Planta* **58,** 664 (1962).
Fryxell, P. A., *Bot. Gaz.* **129**(4), 296 (1968).
Goodwin, T. W., "Modern Methods of Plant Analysis," Vol. 3, Paech, K., Tracey, M. V., Ed., Springer, Heidelberg, 1955, p 272.
Jungalwala, F. B., Cama, H. R., *Biochem. J.* **85,** 1 (1962).
Karrer, P., Rutschmann, J., *Helv. Chim. Acta* **27,** 1684 (1944).
Mackinney, G., *J. Biol. Chem.* **11,** 75 (1935).
Pattee, H. E., Purcell, A. E., *J. Amer. Oil Chem. Soc.* **44,** 328 (1967).
Porter, J. W., Zscheile, F. P., *Arch. Biochem.* **10,** 547 (1946).
Subbarayan, C., Jungalwala, F. B., Cama, H. R., *Anal. Biochem.* **12,** 275 (1965).
Thompson, A. C., Henson, R. D., Hedin, P. A., Minyard, J. P., *Lipids* **3,** 495 (1968).
Zechmeister, L., Sandoval, A., *J. Amer. Chem. Soc.* **68,** 197 (1946).

Barbara W. Hanny*
Rodger D. Henson
Alonzo C. Thompson
Richard C. Gueldner
Paul A. Hedin

Boll Weevil Research Laboratory
Entomology Research Division
U.S. Department of Agriculture
State College, Mississippi 39762

*Received for review December 10, 1971. Accepted January 24, 1972. Part of a dissertation submitted by Barbara W. Hanny in partial fulfillment of requirements for the Ph.D. degree in Botany from Mississippi State University, State College, Miss. Mention of a proprietary product does not necessarily imply endorsement of the product by the U.S. Department of Agriculture.*

# 3,3′,4,4′-Tetrachloroazoxybenzene from 3,4-Dichloroaniline in Microbial Culture

3,3′,4,4′-Tetrachloroazoxybenzene was isolated from cultures of *Fusarium oxysporum* Schlecht growing in the presence of $^{14}$C-ring-labeled 3,4-dichloroaniline. Its identity was established by comparison of its chemical and physical properties with a synthetic sample. Mass spectra, infrared, and ultraviolet spectral properties were determined. It is probable that azoxybenzene is formed by oxidative condensation of two molecules of 3,4-dichlorophenylhydroxylamine or by the condensation of 3,4-dichlorophenylhydroxylamine with 3,4-dichloronitrosobenzene which are potential intermediates in the oxidation of 3,4-dichloroaniline to 3,4-dichloronitrobenzene.

Several condensation products have been isolated from soils treated with aromatic amine compounds. Bartha and Pramer (1967) demonstrated that 3,4-dichloroaniline (3,4-DCA) from 3,4-dichloropropionanilide (propanil) was converted to 3,3′,4,4′-tetrachloroazobenzene (TCAB) in Nixon sandy loam soil. Position of chlorine substituents as well as several other factors was found to affect azobenzene formation (Bartha *et al.*, 1968). Plimmer *et al.* (1970) identified 1,3-bis(3,4-dichlorophenyl)triazene from propanil-treated soils. They proposed that soil nitrite could react with 3,4-DCA to form an intermediate diazonium cation, which would react with another molecule of free aniline to produce the triazene. Condensations of 3-chloraniline and 3,4-DCA to form 3,3′,4′-trichloroazobenzene in addition to 3,3′-dichloroazobenzene and TCAB have also been observed in soil (Kearney *et al.*, 1969). Combinations of propanil and *N*-[3-chloro-4-methylphenyl]-2-methylpentanamide (solan) applied to soil were transformed to TCAB, 3,3′-dichloro-4,4′-dimethylazobenzene, and the asymmetrical 3,3′,4-trichloro-4′-methylazobenzene (Bartha, 1969). The condensation of three molecules of 3,4-DCA to form 4-(3,4-dichloroanilino)-3,3′,4′-trichloroazobenzene has also been reported (Rosen *et al.*, 1969; Linke and Bartha, 1970).

The formation of azobenzenes and anilinoazobenzene has been examined *in vitro* with the aid of horseradish peroxidase (Bartha *et al.*, 1968; Knowles *et al.*, 1969; Lieb and Still, 1969). Few attempts have been made to examine directly the microbial metabolism of halogenated aniline molecules. The formation of azo compounds and related metabolites by soil microorganisms should be examined in greater detail, since anilines are degradation products of many pesticides in soil. The present paper describes the isolation, identification, and probable *in vivo* synthesis of 3,3′,4,4′-tetrachloroazoxybenzene from 3,4-DCA by the soil fungus *Fusarium oxysporum* Schlecht.

## EXPERIMENTAL

Mass spectra were obtained with a Perkin-Elmer Model GC 270 combination gas chromatograph–mass spectrometer using direct or gc inlet systems. The gc column used here was a 50-ft surface-coated open tubular column (SCOT) of