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THE NATURE AND BIOSYNTHESIS OF THE CAROTENOIDS OF DIFFERENT COLOUR VARIETIES OF *CAPSICUM ANNUUM*

B. H. DAVIES, SUSAN MATTHEWS and J. T. O. KIRK*

Department of Biochemistry and Agricultural Biochemistry, University College of Wales,
Aberystwyth, Wales.

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Abstract—The carotenoids of the white-, yellow-, orange- and red-fruiting varieties of the ornamental pepper (*Capsicum annuum*) have been investigated and a scheme for their biosynthesis is discussed.

INTRODUCTION

SOME preliminary studies of the carotenoid content of fruits of different colour varieties of the small ornamental pepper (*Capsicum annuum*) were carried out by Kirk and Juniper,[1] who related the differences in total carotenoid levels in white, yellow, orange and red peppers to comparisons of chromoplast ultrastructure. The carotenoids of the larger fruited varieties of *Capsicum* have been studied in detail, but only in the red[2–4] and yellow[2,5] varieties. The present studies were initated both to extend the observations of Kirk and Juniper and to investigate the possibility that the orange-fruited variety might have some new carotenoids which could be intermediates in the formation of the red carotenoids (e.g. capsanthin and capsorubin) from the carotenoids typical of yellow fruit.

RESULTS

The absorption spectra of the unsaponifiable extracts of *Capsicum* leaves all show the same qualitative absorption maxima in diethyl ether at 447 and 474 nm with an inflexion at 426 nm. Quantitative estimations of the total carotenoid show only a slight variation in total carotenoid content among the four varieties (Table 1). The leaves from the orange-fruited variety have the lowest carotenoid content (13·9 mg/100 g) while those from the red-fruited variety have the highest (24·8 mg/100 g).

The total carotenoid levels of the unripe fruit, however, are lower in all cases by a factor of approximately 40 than those in the leaves (Table 1). The absorption spectra of the three unsaponifiable extracts examined were similar to those of the leaf pigments, each showing absorption maxima at 447 and 474 nm and an inflexion at 426 nm. The fact that all have an identical carotenoid composition was readily demonstrated by TLC on silica gel G.

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* Present address: Commonwealth Scientific and Industrial Research Organisation, Division of Plant Industry, P.O. Box 109, Canberra City, A.C.T., Australia.

[1] J. T. O. KIRK and B. E. JUNIPER, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. II, p. 691, Academic Press, London and New York (1967).

[2] L. CHOLNOKY, K. GYÖRGYFY, E. NAGY and M. PANCZÉL, *Vegyipari Kutatóintézetek Közlemenyei* **4**, 225 (1954).

[3] L. CHOLNOKY, K. GYÖRGYFY, E. NAGY and M. PANCZÉL, *Acta Chim. Hung.* **6**, 143 (1955); *Nature* **178**, 410 (1956).

[4] A. L. CURL, *J. Agr. Food Chem.* **10**, 504 (1962).

[5] L. CHOLNOKY, K. GYÖRGYFY, E. NAGY and M. PANCZÉL, *Acta Chim. Hung.* **16**, 227 (1958).

TABLE 1. TOTAL CAROTENOID CONTENT (mg per 100 g fr. wt.) OF TISSUES FROM RED-, ORANGE-, YELLOW- AND WHITE-FRUITING VARIETIES OF *Capsicum annuum*

	Leaves	Unripe fruit	Ripe fruit
Red-fruiting variety	24·8	0·62	85·5
Orange-fruiting variety	13·9	0·47	2·49
Yellow-fruiting variety	20·8	0·46	2·24
White-fruiting variety	20·9	—	0·069

Analysis of the ripe fruits shows considerable differences, both qualitative and quantitative, between the four varieties. The absorption spectrum in diethyl ether of the unsaponifiable carotenoid extract from the yellow fruit exhibits maxima at 445 (main band) and 469 nm and an inflexion at 423 nm. It is thus similar in appearance to those of the carotenoids from unripe fruit. The absorption spectra of the unsaponifiable extracts from orange and red fruit, however, are qualitatively different from that of the yellow fruit (Fig. 1). While they retain an inflexion at 425 nm and a peak at 449 nm, the main absorption band is at 474 nm and an additional inflexion appears at 501 nm. These two spectra are qualitatively the same and indicate the presence of red carotenoids, presumably capsanthin and capsorubin, in addition to yellow pigments in both the red and the orange fruit.

The quantitative values for the total carotenoid content of the ripe fruit (Table 1) indicate that, in the orange and yellow fruit, carotenoid synthesis takes place during ripening as there is a 5-fold increase in the percentage of carotenoid in the fruit. The increase in the red fruit during ripening is much greater, over 100-fold, leading to a concentration of 85 mg carotenoid per 100 g tissue in the ripe red fruit.

Extracts of the white fruit have no absorption in the visible region of the spectrum but do, in common with yellow, orange and red fruit, show u.v. absorption in the 260–300 nm region. The possibility existed that, in the white fruit at least, such absorption might be due to the presence of phytoene and phytofluene. This was investigated by chromatographing the unsaponifiable material from the fruits on columns of alumina and examining the appropriate fractions for the presence of these carotenoid precursors. Neither of these was detected in any of the differently coloured fruits examined, indicating that the u.v. absorption was due to non-carotenoid substances, presumably including capsaicin. These observations are in contrast to those obtained on chromatography of the unsaponifiable fraction from the larger commercial peppers when phytoene (λ_{max} in light petroleum at 275, 285 and 296 nm), phytofluene (λ_{max} in light petroleum at 331, 347 and 367 nm) and ζ-carotene (λ_{max} in light petroleum at 378, 400 and 425 nm) were clearly detected.

The overall results of the identification tests on the individual carotenoids isolated from the various types of fruit of the ornamental pepper are shown in Table 2. Because of the small quantities of pigment available from these small fruits, only the techniques of spectrophotometry and TLC, together with the associated epoxide tests, could be used. β-Carotene, lutein, zeaxanthin, antheraxanthin, violaxanthin, capsanthin, capsorubin and neoxanthin were identified conclusively on the basis of their properties being identical with those of pure authentic compounds. The monohydroxy-carotenes, cryptoxanthin and hydroxy-α-carotene, were identified by the fact that their absorption maxima in light petroleum were identical with those of zeaxanthin (3,3′-dihydroxy-β-carotene) and lutein (3,3′-dihydroxy-α-carotene) respectively but their adsorption affinities on column and TLC were consistent with their having only one hydroxy group in each. It was not possible to assign a specific structure to

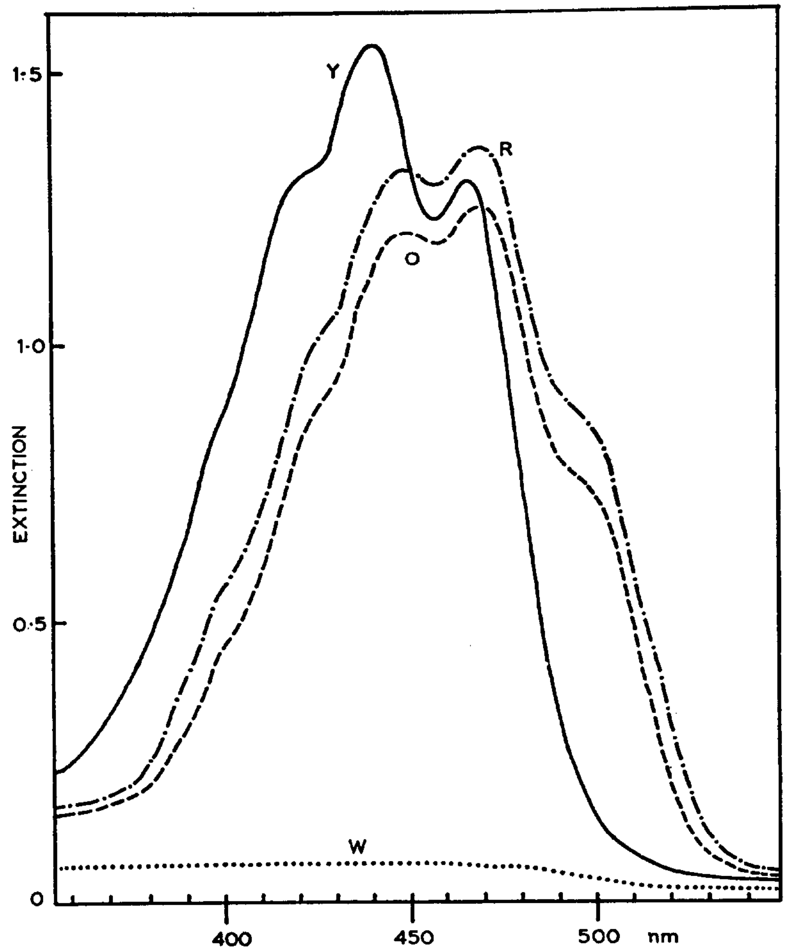

FIG. 1. VISIBLE ABSORPTION SPECTRA (IN DIETHYL ETHER) OF THE UNSAPONIFIABLE LIPID FRACTIONS OF RIPE FRUITS OF *Capsicum annuum*.

Red fruit	—·—·—
Orange fruit	------
Yellow fruit	———
White fruit	....

the hydroxy-α-carotene, but it is more likely to be 3′-hydroxy-α-carotene (α-cryptoxanthin)[6] than the isomeric 3-hydroxy-α-carotene (zeinoxanthin). The pigment identified as β-carotene-5,6-epoxide had a polarity and absorption maxima in light petroleum consistent with this structure; the presence of a 5,6-epoxide was readily demonstrate by the HCl-initiated hypsochromic shift (15 nm) of its absorption maxima in ethanol and by the blue-green colour produced by exposing a thin-layer chromatogram to HCl. The results of similar tests (a 16 nm hypsochromic shift in ethanol and a blue-green colour on the thin layer) suggested that a pink carotenoid, intermediate in adsorption affinity between capsanthin and capsorubin, was capsanthin-5,6-epoxide.[4] The pigment identified as cryptocapsin had the spectral and adsorptive properties consistent with this structure.[4,7]

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[6] L. CHOLNOKY, J. SZABLOCS and E. NAGY, *Annalen* **616**, 207 (1958).

[7] L. CHOLNOKY, J. SZABLOCS, R. D. G. COOPER and B. C. L. WEEDON, *Tetrahedron Letters* 1257 (1963).

TABLE 2. PROPERTIES AND IDENTIFICATION OF THE CAROTENOIDS FROM *Capsicum annuum* FRUITS

Carotenoid	Solvent used to elute carotenoid from zinc carbonate column	Solvent used to elute carotenoid from calcium carbonate column	R_f†	Absorption maxima in light petroleum (nm)	Absorption maxima in benzene (nm)	Hypsochromic shift in ethanol with HCl	Colour given by carotenoid with HCl on TLC plate
β-Carotene	Lt. pet.	—	1·0	425 449 477		0	Yellow-brown
β-Carotene-5,6-epoxide	Lt. pet.	—	0·95	— 446 476		15	Blue-green
Hydroxy-α-carotene	2% e/p*	—	0·72	421 446 475		0	Yellow-brown
Cryptoxanthin	2% e/p	—	0·70	425 449 476		0	Yellow-brown
Cryptocapsin	5% e/p	—	0·64	— 475 505	— 483 518	0	Brown
Lutein	20% e/p	—	0·57	421 445 474		0	Brown and green
Zeaxanthin	20% e/p	—	0·53	424 449 476		0	Brown and green
Antheraxanthin	50% e/p	—	0·40	422 445 472		16	Blue-green
Violaxanthin	Ether	5% e/p	0·35	418 442 466		39	Blue
Capsanthin	Ethanol	10% e/p	0·29	(450)475 505	(460)483 518	0	Brown
Capsanthin-5,6-epoxide	Ethanol	20% e/p	0·24	— 472 —	— 479 513	16	Blue-green
Capsorubin	Ethanol	20% e/p	0·20	(455)479 510	(460)488 523	0	Brown
Neoxanthin	Ethanol	25% e/p	0·15	418 442 467		17	Blue-green

* % e/p = Percentage of diethyl ether in light petroleum.

† TLC on silica gel G developed with benzene–EtOAc–MeOH (75:20:5 by volume).

The results of the quantitative determinations of individual carotenoids isolated from ripe red, orange and yellow *Capsicum* fruits, and from green, unripe fruit (from red-fruiting plants) are shown in Table 3. The amount of each carotenoid is expressed as a percentage of the.total carotenoid recovered. There were traces of *cis* isomers of many of the carotenoids, but these have been ignored as they were present in quantities too small for a detailed examination.

TABLE 3. THE OCCURRENCE OF INDIVIDUAL CAROTENOIDS IN THE FRUITS OF COLOUR VARIETIES OF *Capsicum annuum*

Carotenoid	Ripe red	Ripe orange	Ripe yellow	Unripe red, i.e. green
β-Carotene	12·3	—	1·0	20·3
β-Carotene-5,6-epoxide	3·1	—	—	1·7
Hydroxy-α-carotene	—	—	5·7	—
Cryptoxanthin	7·8	—	—	3·6
Cryptocapsin	5·0	8·0	—	—
Lutein	—	—	28·1	39·7
Zeaxanthin	6·5	7·3	—	1·0
Antheraxanthin	9·2	10·6	15·9	5·0
Violaxanthin	9·8	14·9	31·3	13·3
Capsanthin	31·7	35·4	—	—
Capsanthin-5,6-epoxide	4·2	6·1	—	—
Capsorubin	7·5	14·3	—	—
Neoxanthin	2·0	2·7	20·0	15·4

Expressed as the percentage of the total carotenoid recovered.

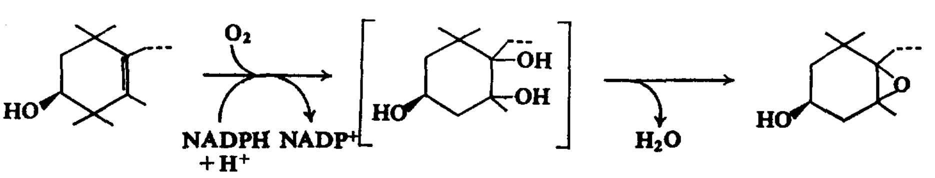
DISCUSSION

The results shown in Tables 1–3 indicate that in the red-fruiting *Capsicum*, the level of carotenoids in the unripe fruit is much lower than that in the leaves, but that it increases by a factor of about 100 during the process of ripening. The yellow variety is similar to the red in that the total carotenoid content is less in the unripe fruit than in the leaves, but the increase on ripening is only of the order of about 5-fold. The major qualitative differences between these two types of ripe fruit are that the yellow fruit contain lutein (3,3′-dihydroxy-α-carotene) and violaxanthin (3,3′-dihydroxy-5,6,5′,6′-diepoxy-β-carotene) as the major carotenoids, together with other similar xanthophylls, while in the red fruit, the major carotenoids are the characteristic "paprika ketones", capsanthin and capsorubin; lutein is completely absent from the ripe red fruit. These results merely confirm the observations of Cholnoky and his collaborators.[2,3,5]

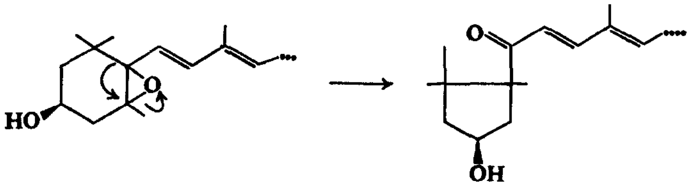
The increase in the total carotenoid of the orange fruit during ripening is almost identical with that in the yellow variety, but the carotenoids of these two types of ripe fruit are qualitatively different. The carotenoids of the orange fruit are virtually identical with those of the ripe red fruit, except that the less oxygenated carotenoids, β-carotene, β-carotene-5,6-epoxide and cryptoxanthin, are absent. Perhaps this is a reflection of the fact that the orange fruit do not have nearly as efficient a *de novo* carotenoid synthesizing system as the red fruit, so that the small quantities of these precursors that are formed are used up immediately. The absence of the early carotene precursors, phytoene, phytofluene and ζ-carotene from these small ornamental peppers, in contrast to their presence in the ripe fruit of the larger commercial pepper, suggests that the latter have either a more efficient *de novo* synthesizing system or a less efficient oxygenating system than the small fruit.

As the total carotenoid levels of the orange and yellow fruit are so similar, it is possible to make more realistic comparisons of the levels of individual carotenoids than is the case with the red fruit. The results (Table 3) are consistent with the formation of red keto-carotenoids with acylcyclopentanol end groups[8] from the carotenoid epoxides. The absence of any possible intermediates from the orange fruit suggests that this step is direct. The enzyme which is responsible for this conversion, and which normally appears during ripening, must be absent from the yellow fruit. The utilization of lutein during ripening is probably indirect, requiring the intermediate formation of a 5,6-epoxide. A possible biosynthetic sequence for the formation of the red ketocarotenoids from β-carotene is shown in Fig. 2.

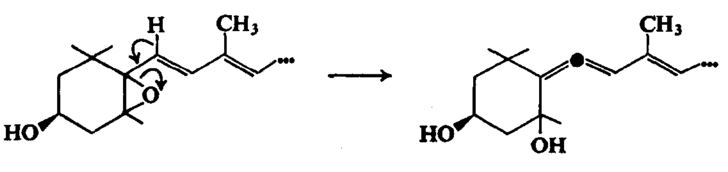
The conversion of zeaxanthin into antheraxanthin and violaxanthin that takes place in other higher plants[9] (and, presumably, in *Capsicum* fruits) uses molecular oxygen and can be visualized as a mixed function oxidation with the elimination of a molecule of water.



There seems little doubt that the five-membered ring characteristic of capsanthin, capsanthin-5,6-epoxide, cryptocapsin and capsorubin is formed from a 3-hydroxy-5,6-epoxy end group by a pinacollic rearrangement leading to the acylcyclopentanol structure.[10] The enzyme which catalyses this step is obviously absent from the yellow fruit.



An alternative transformation which might occur is the rearrangement of the 3-hydroxy-5,6-epoxy end group to form the allenic 3,5-dihydroxy end group which is characteristic of neoxanthin; neoxanthin has the 3-hydroxy-5,6-epoxy-β-ionone group at the other end of the molecule.[11]



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[8] R. ENTSCHEL and P. KARRER, *Helv. Chim. Acta* **43**, 89 (1960); M. S. BARBER, L. M. JACKMAN, C. K. WARREN and B. C. L. WEEDON, *Proc. Chem. Soc.* **19** (1960); *J. Chem. Soc.* 4019 (1961).

[9] H. YAMAMOTO and C. O. CHICHESTER, *Biochim. Biophys. Acta* **109**, 303 (1965).

[10] H. FAIGLE and P. KARRER, *Helv. Chim. Acta* **44**, 1257 (1961); B. C. L. WEEDON, *Chemistry in Britain* **3**, 424 (1967).

[11] L. CHOLNOKY, K. GYÖRGYFY, A. RONAI, J. SZABLOCS, G. TOTH, G. GALESKO, A. K. MALLAMS, E. S. WAIGHT and B. C. L. WEEDON, *J. Chem. Soc.* (C), 1256 (1969).

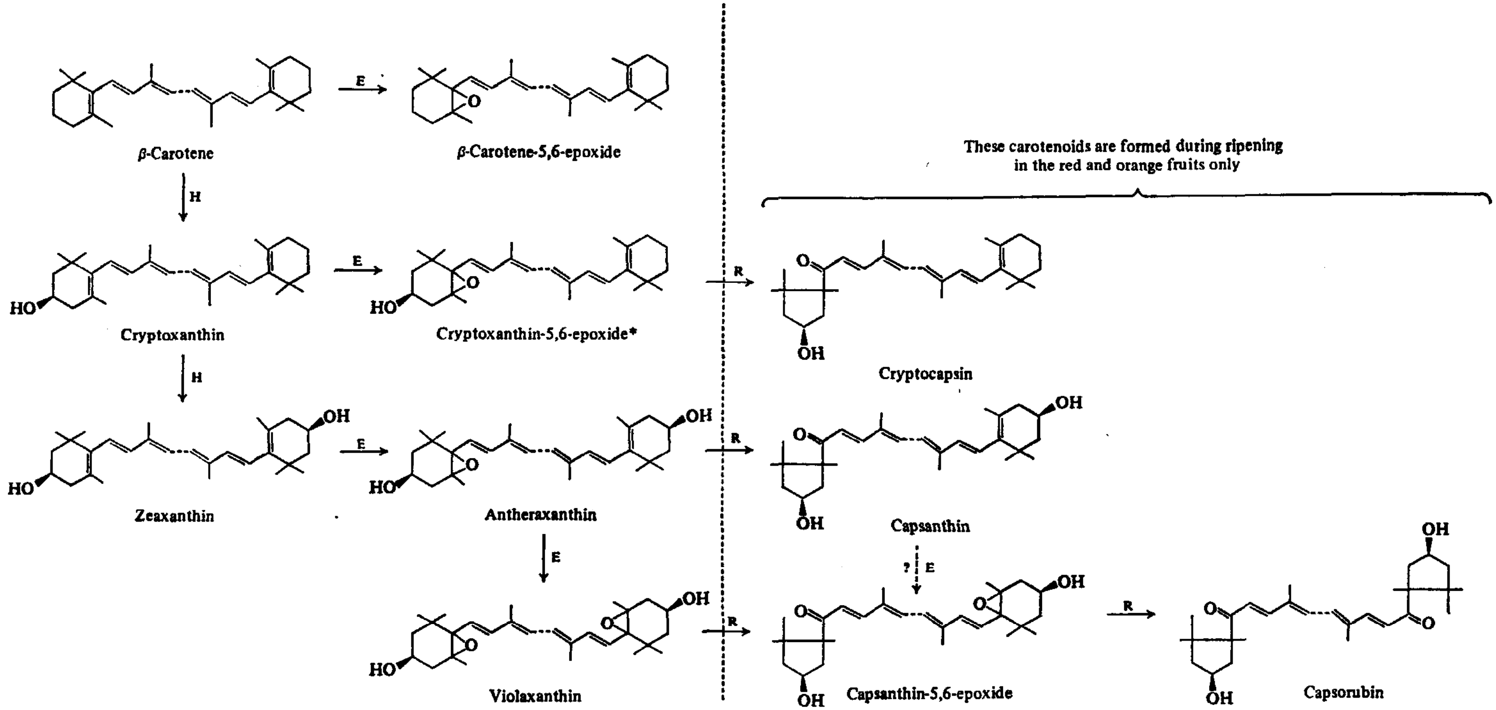

FIG. 2. BIOSYNTHESIS OF RED KETOCAROTENOIDS IN FRUIT OF *Capsicum annuum* (AFTER CHOLNOKY *et al.*[3]).

H = Hydroxylation
E = Epoxidation
R = Rearrangement
- - - - Biosynthetic lesion in yellow fruit
* Not detected

Neoxanthin appears at a relatively high concentration in the ripe yellow *Capsicum* fruit. Perhaps it represents the final product of xanthophyll interconversions, only occurring to any appreciable extent when the enzyme forming the paprika ketones is absent. Another enzyme that is absent from the yellow fruit is that responsible for the disappearance of lutein during ripening in the orange and red fruit.

At present, there is no evidence to indicate that the formation of the red ketocarotenoids is anything but irreversible. On the other hand, the reversibility of the epoxide-forming steps has been demonstrated by other workers and is the basis for a mechanism of oxygen transport mediated by the xanthophylls of photosynthetic tissue.[3, 12]

EXPERIMENTAL

Materials

Three of the colour varieties, red, orange and yellow, were obtained from S. Dobie and Son, Ltd., Chester, under the name "Ornamental Pepper (Capsicum)—Tall Mixed". The white variety was kindly provided by Mr. J. K. Burras, Superintendent of the Oxford Botanic Garden. The plants were grown from seed in a greenhouse and the fruits taken at the appropriate stage. The large ripe red pepper fruits were bought locally.

Authentic Carotenoids

The carotenoids of the large ripe commercial peppers have been studied in detail[3, 4] and their properties, together with those of other higher plant carotenoids, are well documented.[13] Thus, chromatographically pure samples of capsanthin, capsorubin and antheraxanthin were prepared, using the methods described below, from locally bought red peppers. Samples of lutein, violaxanthin and neoxanthin were prepared from extracts of the leaves of young maize seedlings. Crystalline samples of β-carotene and zeaxanthin were kindly provided by F. Hoffmann-La Roche and Co., Basel, Switzerland.

Purification of Solvents

All the solvents were of Analar grade. Light petroleum (40–60°) and diethyl ether were dried over Na and redistilled from reduced iron powder prior to use. Benzene was dried over Na, while methanol was distilled from KOH.

Extraction of Carotenoids

The individual fruits (the small ornamental peppers weighing 2 to 5 g and the large red fruits weighing about 100 g each) were cut open and the seeds removed and discarded. The remainder of each fruit was weighed and then homogenized in cold acetone. The mixture was filtered with a sintered-glass funnel and the residue repeatedly extracted with more acetone until the filtrate was colourless. The lipid was transferred to freshly distilled diethyl ether by the addition of distilled water and the ether solution washed several times with distilled water before the solvent was removed under reduced pressure and the wet residue dissolved in ethanol and saponified with potassium hydroxide in order to remove chlorophyll and saponifiable lipids.[13] The unsaponifiable fraction was extracted with ether, washed free of alkali with distilled water and dried over anhydrous sodium sulphate. The total carotenoid was estimated at this stage, prior to the ether being distilled off under a stream of nitrogen and the residue dissolved in a small volume of freshly distilled light petroleum prior to chromatography. While the estimations of the total carotenoid contents were carried out on individual fruits, the extracts from several fruits of the same type were combined for the chromatographic separation of the pigments.

Separation of Carotenoids

The carotenoids of the small *Capsicum* fruit samples were separated on columns (10 × 1 cm) of zinc carbonate/Hyflo-Super-Cel (1:1, w/w), developing the columns with light petroleum containing increasing concentrations (up to 100%) of diethyl ether; the separate coloured fractions were collected as they were

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[12] N. I. Krinsky, in *Photophysiology, Current Topics* (edited by A. C. Giese), Vol. III, p. 123, Academic Press, New York and London (1968).

[13] B. H. Davies, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. Goodwin), p. 489, Academic Press, London and New York (1965).

eluted. Any carotenoids remaining on the column after this treatment were eluted with absolute ethanol, transferred to light petroleum and rechromatographed on columns of calcium carbonate/Hyflo-Super-Cel (1:1, w/w) using increasing concentrations of diethyl ether in light petroleum as the developing solvent. The individual carotenoids from these two preliminary separations were further purified on smaller columns (7 × 0·5 cm) of either zinc carbonate/Hyflo-Super-Cel (2:1, w/w) or calcium carbonate/Hyflo-Super-Cel (2:1, w/w), as appropriate. The eluting solvent, which passed through the column under pressure, was the appropriate concentration of diethyl ether in light petroleum. Details of these solvents are given in Table 2.

For the preparation of the samples of authentic carotenoids, the same chromatography systems were employed, but on a larger scale. The presence of phytoene, phytofluene and ζ-carotene in the unsaponifiable fraction of the commercial peppers was indicated by the characteristic absorption spectra of the fractions eluted from an alumina column (Woelm neutral, Brockmann activity grade II) with light petroleum and 1% diethyl ether in light petroleum.

Identification of Carotenoids

The absorption spectra of the pure carotenoids were recorded in light petroleum or in benzene. Very small samples were examined in 0·5 ml microcells in the S.P. 800 spectrophotometer, using the scale expansion output to drive a Beckman 100504U slave recorder.

Carotenoid epoxides were identified by observing the magnitude of the spectral shift on adding a small drop of conc. HCl to an ethanolic solution of the carotenoid in a spectrophotometer cell.[13] Mono-5,6-epoxides were characterized by a hypsochromic shift of 15–20 nm, while 5,6,5′,6′-diepoxides showed a shift of about 40 nm.

TLC of the carotenoids was used solely as a method of identification, and was carried out in a saturation chamber[14] on 250 μm layers of silica gel G (Merck), developing with benzene–EtOAc–MeOH (75:20:5) by volume. Wherever possible, samples of authentic carotenoids were cochromatographed with the individual pigments from the *Capsicum* fruits in order to make conclusive identifications.

Epoxides were also characterized by their reaction with HCl on the thin layers of silica gel G. The developed plates were exposed, in a fume cupboard, to a stream of air which had been passed through conc. HCl contained in a Dreschel bottle fitted with a sintered distributor. The diepoxides gave a rich blue colour on this treatment while the monoepoxide zones turned blue-green. Dihydroxy-carotenoids gave an unusual but completely reproducible effect in that they yielded brown zones which were bordered with green. The other carotenoids gave no more than a yellow to brown colour.

Quantitative Determination of Carotenoids

All the quantitative determinations of carotenoids were carried out spectrophotometrically in 1 cm path length quartz cells or micro-cells on solutions of known volume.[13] The $E^{1\%}_{1\,\mathrm{cm}}$ value for the yellow carotenoids in either diethyl ether or light petroleum was assumed to be 2500 while that of the red carotenoids was taken as 1750 in diethyl ether. In the case of mixtures of red and yellow carotenoids (in the total extracts of ripe orange and red peppers), an approximate value of 2000 was used for the $E^{1\%}_{1\,\mathrm{cm}}$ in diethyl ether.

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[14] B. H. Davies, *J. Chromatog.* **10**, 518 (1963).