BIOSYNTHESIS OF KETO-CAROTENOIDS IN *CAPSICUM ANNUUM* FRUITS

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1. Introduction

During the ripening of *Capsicum annuum*, there is a massive synthesis of keto-xanthophylls (keto-group at C_6). These xanthophylls (fig.1) include Capsanthin (I), capsorubin (II) and cryptocapsin (III). A hypothesis about their biosynthesis has been proposed in [1–4].

This report deals with our experiments and provides more detailed information on the subject. Results obtained from the biosynthesis of squalene [5] and carotenoids [6] with a stereospecifically labelled substrate show that the H_S of mevalonate is lost while the H_R is retained at the C_4 level of the molecule. From this, we were able to demonstrate that capsanthin, capsorubin and cryptocapsin are biosynthesized in the same way as β-carotene or xanthophyll with a β-epoxy-cyclohexenyl group. This result is sustained by a comparison of the molar specific radioactivity of [^{14}C]capsanthin and β-[^{14}C]citraurin obtained after alkaline degradation.

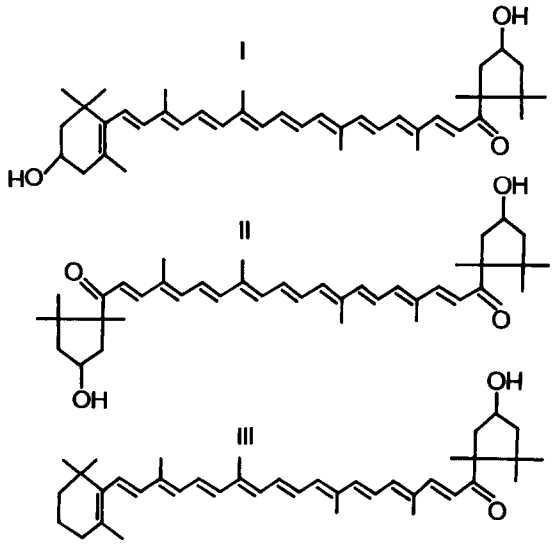

Fig.1. Structures of the different keto-xanthophylls: (I) capsanthin; (II) capsorubin; (III) cryptocapsin.

2. Materials and methods

2.1. *Incubation conditions*

[2-^{14}C]- and [3*R*,4*R*-4-^{3}H + 3*S*,4*S*-^{3}H]Mevalonic acid lactones were obtained from the Radiochemical Center Amersham. They were converted to the potassium salt before use. The resulting solution was adjusted to pH 7.6 with 0.1 M Tris buffer.

The labelled substrate was applied as microdroplets to 60 disks excised from the semi-ripened pericarp tissue of *Capsicum annuum* fruits (196 μCi [^{3}H]-mevalonate and 36 μCi [^{14}C]mevalonate for double labelling experiments and 200 μCi [^{14}C]mevalonate for single labelling experiments). The disks were incubated with 10 ml 0.1 M Tris buffer (pH 7.6) at 25°C and 4300 lux. After 26 h, they were washed with cold water and chilled in liquid nitrogen.

2.2. *Extraction, identification and purification of carotenoids*

The pericarp disks were extracted with cold acetone at 0–4°C under a dim light. The saponified extract was chromatographed on thin layers of Silica gel G (adjusted with KOH to pH 7) with 40% acetone in petroleum ether (b.p. 40–60°C). The identification procedures were described in [7]. The bands containing xanthophylls were eluted and chromatographed on thin layers of MgO–Kieselgur G (1:1)

with 40% acetone in petroleum ether. A further purification was performed on thin layers of Silica gel G, with 30% ethyl acetate in carbon tetrachloride for monohydroxylated xanthophylls and with 30% carbon tetrachloride in ethyl acetate for dihydroxylated xanthophylls. The constant specific radioactivity obtained was ascertained by further acetylation in pyridine–acetic anhydride [8] or acid isomerization of the 5–6 epoxy-group with 2% HCl. The band containing carotenes was subjected to a preliminary purification on thin layers of Silica gel G with petroleum ether. The carotenes were rechromatographed on thin layers of MgO–Kieselgur (1:1) with 10% benzene in petroleum ether. Phytoene, *cis*-phytofluene, *trans*-phytofluene, α-carotene were eluted and subjected to a further purification on Silica gel G plates with 5% benzene in petroleum ether. β-carotene and ζ-carotene were separated on Siliga gel with petroleum ether and purified on Silica gel with 5% benzene in petroleum ether.

2.3. *Alkaline hydrolysis of capsanthin*

Capsanthin (20 mg) was isolated from the pericarp tissue of *Capsicum* fruits by a combination of various methods: phase-partition [8]; column chromatography on cellulose powder [9]; and preparative chromatography on Silica gel plates and MgO–Kieselgur G (1:1) plates with 40% acetone in petroleum ether. After crystallization in benzene–methanol, the identity of the product was confirmed by mass spectra: molecular ion M^+ at *m/e* 584 and fragment ions at *m/e* 566 (M-18) loss of water; *m/e* 492 (M-92) loss of toluene; *m/e* 478 (M-106) loss of xylene; *m/e* 460 (M-124) loss of xylene and water; and *m/e* 429 (M-155). The ratio of the intensity of M-92/M-106, 0.1 was close to that in [10]. The unlabelled capsanthin was mixed with [^{14}C]capsanthin to give spec. radioact. 153 000 dpm/mmol. The product was then subjected to alkaline hydrolysis under nitrogen atmosphere, by the procedure in [11]. A preliminary separation was performed on Silica gel plates (2 mm) with 30% petroleum ether in ethyl ether as developing solvent. The band containing β-citraurin was localized using a genuine sample. This fraction was purified on Silica gel plates with 30% carbon tetrachloride in ethyl acetate. A further purification was performed on a Silica gel plate with 40% benzene in petroleum ether. The resulting β-citraurin was crystallized in benzene–methanol yielding dark orange crystals, (λ_{max} were 450–476 nm in hexane, and 467–497 nm in benzene). The identity of this product was confirmed by mass spectra molecular ion M^+ at *m/e* 432 and fragment ions at *m/e* 326 (M-106) *m/e* 308 (M-124). An aldol condensation of acetone and β-citraurin was carried out [12] as an alternative for the identification procedure. Under these conditions, reticulataxanthin λ_{max} (benzene) 458–505 nm was formed.

2.4. *Radioactivity determination*

The carotenoids were dissolved in the scintillation fluid of [13]. ^{14}C and ^{3}H were counted simultaneously with the following efficiencies ^{14}C (70%), ^{3}H (38%) and ^{14}C in the ^{3}H channel (11%); the ^{3}H in ^{14}C channel was negligible. Correction for quenching was made by an automatic external standardization method [14]. The results obtained were the average of 6 radioactivity determinations.

3. Results and discussion

As established [15] the sequential desaturation which occurred did not affect hydrogen at the C_4 level of mevalonate. Therefore, the atomic ratios $^{3}H/^{14}C$ of the uncyclized precursors (phytoene, phytofluene and ζ-carotene) were similar and close to 8:8 (table 1). On the other hand, the main change occurred at the cyclization level. The 4 *pro R* hydrogen of mevalonic acid was lost from the C_6 of the β-cyclohexenyl ring. For β-carotene (the first bi-cyclic carotenoid) the ratio was close to 6:8. The same ratio was also observed for β,β-cyclic xanthophylls (β-cryptoxanthin, zeaxanthin, antheraxanthin, violaxanthin) as well as for β,κ-cyclic xanthophyll (capsanthin and cryptocapsin) or κ,κ-cyclic xanthophyll (capsorubin). Elimination of the 4 *pro R* hydrogen of mevalonic acid occurred during the biogenesis of the κ-ring of the keto-xanthophylls. One can thus conclude that capsanthin, capsorubin and cryptocapsin are biosynthesized in the same way as β-carotene. The ratios obtained when considered in relation to the fact that xanthophyll hydroxylation takes place at a final step of the biosynthetic pathway [16] as well as the presence of violaxanthin of the appearance of antheraxanthin during the ripening period [4], support the hypothesis that keto-xanthophylls in *Capsicum* fruits are biosynthesized via cyclohexenyl-epoxide as was demonstrated in [14]. The mechanism suggested involves a pinacolic rearrangement [17–20] according to the scheme in fig.2.

Table 1
Incorporation of [2-^{14}C, 4*R*-4-$^{3}H_1$]mevalonate into carotenoids of *Capsicum annuum* fruits

Carotenoids	^{3}H (dpm)	^{14}C (dpm)	^{3}H (dpm) / ^{14}C (dpm)	^{3}H/^{14}C atomic ratios[a]	
				Observed	Theoretical
Phytoene	204 990	27 821	7.36		
cis-Phytofluene	40 010	5465	7.32	7.95:8	8:8
trans-Phytofluene	1376	190	7.24	7.83:8	8:8
ζ-Carotene	22 721	3150	7.21	7.83:8	8:8
β-Carotene	240 660	43 600	5.51	5.98:8	6:8
β-Cryptoxanthin	30 055	5480	5.48	5.95:8	6:8
Cryptocapsin	9310	1745	5.33	5.79:8	6:8
Zeaxanthin	22 850	4200	5.44	5.91:8	6:8
Antheraxanthin	52 240	9532	5.48	5.95:8	6:8
Violaxanthin	77 247	14 173	5.45	5.92:8	6:8
Capsanthin	82 305	15 101	5.45	5.92:8	6:8
Capsorubin	13 585	2506	5.42	5.89:8	6:8

[a] ^{3}H/^{14}C atomic ratios based on a ratio of 8:8 for phytoene

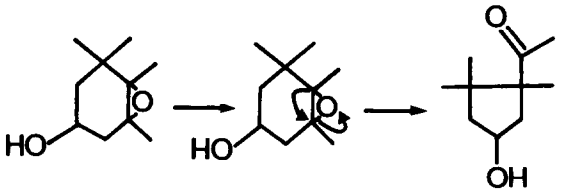

Fig.2. Possible pathway for the biogenesis of the keto-carotenoids.

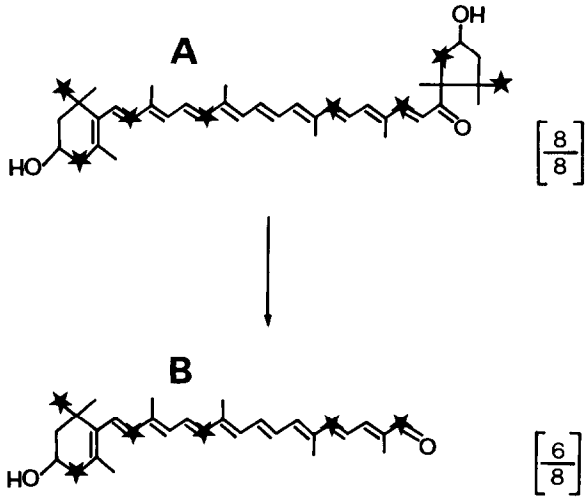

Fig.3. Expected labelling of capsanthin (A) and β-citraurin (B) from [2-^{14}C]mevalonate. (⋆) Represents the ^{14}C atoms of [2-^{14}C]mevalonate incorporated. Figures in brackets represent the relative specific radioactivity.

Capsanthin obtained from [2-^{14}C]mevalonic acid had spec. radioact. 153 000 dpm/nmol. Based on uniform incorporation, this represents a ratio of 8:8. On the other hand for β-citraurin the ratio is 6:8 (fig.3). Therefore the specific radioactivity of β-citraurin must be 75% that of capsanthin. The results presented in table 2 correlates with the theoretical value; these data and the above results support the hypothesis that in *Capsicum* fruits the biosynthesis of the keto-xanthophylls develops by a rearrangement of the epoxy-cyclohexenyl group of β,β-xanthophylls.

Acknowledgements

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Table 2
Comparison of the specific radioactivities of [^{14}C]capsanthin and β-[^{14}C]citraurin obtained after alkaline hydrolysis of the former

Carotenoids	dpm/mmol	Ratio[a]
Capsanthin	153 000	100
β-Citraurin	113 900	74.5

[a] Ratio relative to capsanthin (%)

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