Biochem. J. (1969) **112**, 383
Printed in Great Britain

Biosynthesis of Xanthophylls in Higher Plants: Stereochemistry of Hydroxylation at C-3

By T. J. WALTON, G. BRITTON and T. W. GOODWIN
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool

(*Received* 20 *January* 1969)

A number of problems in the biosynthesis of carotenoids have been investigated with various species of doubly labelled MVA* (Williams, Britton, Charlton & Goodwin, 1967*a*; Williams, Britton & Goodwin, 1967*b*). This work has enabled the stereochemistry of formation of phytoene, the probable first C_{40} intermediate in carotene biosynthesis, to be established in a number of systems. In addition, the stereochemistry of the introduction of double bonds informing more unsaturated pigments, e.g. lycopene and β-carotene, has been studied, and evidence obtained showing that α- and β-ionone rings in carotenes are formed independently. These studies were confined to the carotene hydrocarbons, but the development of modified purification techniques has now enabled our investigations to be extended to cover the oxygenated carotenoids, i.e. xanthophylls. In particular, one major outstanding problem, the stereochemistry of introduction of hydroxyl functions at C-3 and C-3′ of cyclic carotenoids, has been studied by the use of [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA and [2-^{14}C,5-$^{3}H_2$]MVA.

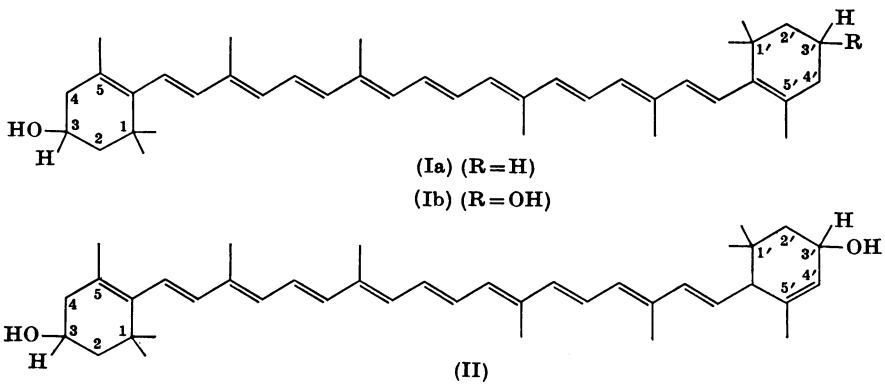
Materials and methods. Etiolated maize seedlings (*Zea mays*, Giant Hybrid, White Horsetooth) were excised, and incubated in the light at 22° for 28 hr. with their stems immersed in 0·1M-tris–HCl buffer, pH 7·4, containing [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA (potassium salt, 10μc of ^{14}C, 100μc of ^{3}H) or [2-^{14}C,5-$^{3}H_2$]-MVA (potassium salt, 9·6μc of ^{14}C, 96μc of ^{3}H). Sliced cups and berries of *Physalis alkekengi* were incubated with [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA (potassium salt, 5 μc of ^{14}C, 40 μc of ^{3}H) or [2-^{14}C,5-$^{3}H_2$]MVA (potassium salt, 4·8 μc of ^{14}C, 48 μc of ^{3}H) in 0·1 M-tris–HCl buffer, pH 7·4, for 48 hr. at 25° in the dark. Pigments were extracted and saponified by the normal method (Britton & Goodwin, 1969). In each case, the extract was chromatographed on a column of neutral alumina (Brockmann grade III); carotenes were eluted with 0·5% (v/v) E/P, monohydroxy compounds with 60% (v/v) E/P and dihydroxyxanthophylls with 5% (v/v) ethanol/E. The carotenes were purified by successive t.l.c. on (*a*) silica gel G with 0·5% (v/v) E/P, (*b*) MgO–kieselguhr G (1 : 1, w/w) with 12% B/P and (*c*) silica gel G with 5% (v/v) B/P as developing solvents.

* Abbreviations: MVA, mevalonic acid; in solvents: E, diethyl ether; P, light petroleum (b.p. 40–60°); A, acetone; B, benzene.

β-Cryptoxanthin was purified by successive t.l.c. on (*a*) silica gel G with 70% (v/v) E/P, (*b*) MgO–kieselguhr G (1 : 1, w/w) with 25% A/P and (*c*) silica gel G with 2% (v/v) methanol/B as developing solvents. Dihydroxycarotenes were purified by successive t.l.c. on (*a*) silica gel G with 70% (v/v) E/P, (*b*) MgO–kieselguhr G (1 : 1, w/w) with 60% (v/v) A/P and (*c*) silica gel G with 5% (v/v) methanol/B as developing solvents. Portions of the lutein, zeaxanthin and β-cryptoxanthin samples were acetylated (acetic anhydride–pyridine), and the acetates purified by t.l.c. on silica gel G with 8% (v/v) E/P (β-cryptoxanthin) or 20% (v/v) E/P (lutein, zeaxanthin) as developing solvents. Purification methods are described fully elsewhere (Britton & Goodwin, 1969). Samples of the purified products were decolorized by the normal method (Williams *et al.* 1967*a*) and simultaneously assayed for ^{14}C and ^{3}H radioactivity with a Beckman LS 200B liquid-scintillation system. ^{14}C and ^{3}H radioactivities and atomic ratios were calculated by the standard method (Britton & Goodwin, 1969).

As a check on the radiochemical purity of the *Physalis* zeaxanthin, a portion was isomerized by iodine, and the isomers were separated and assayed for ^{14}C and ^{3}H radioactivity. There was no significant difference in the ^{3}H/^{14}C radioactivity ratios of the isomers and the starting material.

Results and discussion. Most xanthophylls occurring commonly in higher plants, e.g. β-cryptoxanthin (Ia), zeaxanthin (Ib) and lutein (II), have hydroxyl functions at C-3 and C-3′ in the rings, these carbon atoms originating from C-5 of MVA. Previous work has shown that the two hydrogen atoms from C-5 of MVA are retained at these positions in the carotene molecule. One or both of these hydrogen atoms must, however, be lost in the formation of 3-hydroxycarotenoids. The loss of hydrogen from this position during the formation of xanthophylls has therefore been studied, by incorporation of [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA and [2-^{14}C,5-$^{3}H_2$]MVA into maize leaves, a source of lutein and zeaxanthin, and into cups and berries of *Physalis alkekengi*, a source of β-cryptoxanthin and zeaxanthin. The results of one series of incubations are given in Table 1. This shows that, in *Physalis*, β-carotene formed from [2-^{14}C,5-$^{3}H_2$]MVA has a ^{14}C/^{3}H atomic ratio 8 : 10, in agreement with earlier



(Ia) (R=H)
(Ib) (R=OH)

(II)

Table 1. *Incorporation of* [2-^{14}C,(5*R*)-5-^{3}H$_1$]*MVA and* [2-^{14}C,5-^{3}H$_2$]*MVA into carotenoids in maize seedlings and in fruit of* Physalis alkekengi

	[2-^{14}C,(5*R*)-5-^{3}H$_1$]MVA				[2-^{14}C,5-^{3}H$_2$]MVA			
Compound	^{14}C radio-activity (d.p.m.)	^{3}H radio-activity (d.p.m.)	^{3}H/^{14}C radio-activity ratio	^{14}C/^{3}H atomic ratio*	^{14}C radio-activity (d.p.m.)	^{3}H radio-activity (d.p.m.)	^{3}H/^{14}C radio-activity ratio	^{14}C/^{3}H atomic ratio†
(1) From maize seedlings								
MVA	3173	36402	11·47	1:0·98	23638	329155	13·92	1:2·04
Squalene	59637	769000	12·89	—	33069	369309	12·84	—
α-Carotene	85	485	5·70	8:3·90	212	1737	8·21	8:9·76
β-Carotene	3780	22126	5·85	8:4·00	4841	41175	8·51	8:10·00
Lutein	3197	9130	2·86	8:1·95	917	6304	6·88	8:8·09
Lutein diacetate	28	83	2·92	8:2·00	1859	12535	6·74	8:7·93
Zeaxanthin	532	584	1·10	8:0·75	192	1196	6·22	8:7·31
Zeaxanthin diacetate	128	95	0·74	8:0·51	208	1276	6·14	8:7·22
(2) From *Physalis alkekengi*								
MVA‡	1433	9895	6·90	1:1·00	—	—	—	—
Phytoene	5466	37918	6·94	8:8·02	3256	31058	9·54	8:13·40
β-Carotene	5442	18779	3·45	8:4·00	2965	21105	7·12	8:10·00
β-Cryptoxanthin	855	2314	2·70	8:3·13	4120	27321	6·63	8:9·32
β-Cryptoxanthin acetate	944	2435	2·58	8:2·98	1754	11435	6·52	8:9·16
Zeaxanthin	1144	244	0·22	8:0·26	4033	21890	5·43	8:7·63
Zeaxanthin diacetate	3499	810	0·23	8:0·27	2919	15953	5·46	8:7·68

* Based on a ^{14}C/^{3}H atomic ratio 8:4 for β-carotene.
† Based on a ^{14}C/^{3}H atomic ratio 8:10 for β-carotene.
‡ The MVA used in this experiment was from a different batch.

results obtained for β-carotene in other systems. β-Cryptoxanthin, however, has a ^{14}C/^{3}H atomic ratio 8 : 9, showing that only one hydrogen atom originally from C-5 of MVA is lost in the introduction of the hydroxyl function. A ^{14}C/^{3}H atomic ratio 8:3 for β-cryptoxanthin formed from [2-^{14}C,(5*R*)-5-^{3}H$_1$]MVA, compared with the ratio 8 : 4 for β-carotene, shows that it is the *pro-R*-hydrogen atom from C-5 of MVA that is lost in the introduction of the hydroxyl group of β-cryptoxanthin, the *pro-S*-hydrogen atom being retained.

In the maize-leaf experiments, lutein biosynthesized in the presence of [2-^{14}C,5-^{3}H$_2$]MVA has a ^{14}C/^{3}H atomic ratio 8 : 8, showing a loss of two labelled hydrogen atoms when compared with α- and β-carotene (^{14}C/^{3}H atomic ratios 8 : 10). The ^{14}C/^{3}H atomic ratios obtained with [2-^{14}C,(5*R*)-5-^{3}H$_1$]-MVA (8 : 4 for α- and β-carotene, 8 : 2 for lutein) show that in this case also one *pro-R*-hydrogen atom from C-5 of MVA is lost in the introduction of each of the hydroxyl functions, and that the corresponding *pro-S*-hydrogen atom is retained.

Thus in the biosynthesis of β-cryptoxanthin and

lutein the hydroxyl functions are introduced by stereospecific replacement of one hydrogen atom at C-3 or C-3′, the hydrogen atom replaced in each case being that which was originally the *pro-R*-hydrogen atom at C-5 of MVA. The results rule out the possibility that hydroxylation proceeds via a keto intermediate, which would require the loss of both hydrogen atoms from C-3. They also rule out the possibility that the hydrocarbons are formed from xanthophylls. The $^{14}C/^{3}H$ atomic ratios obtained for zeaxanthin in both systems are unexpected because essentially all the ^{3}H from [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA is lost. Recent experiments (T. J. Walton, J. C. B. McDermott, G. Britton & T. W. Goodwin, unpublished work) indicate that the stereochemistry of zeaxanthin biosynthesis is more complex than was at first thought.

Physalis alkekengi plants were obtained from the University of Liverpool Botanic Gardens, Ness. We thank the Science Research Council for support, and for a research studentship (to T.J.W.).

Britton, G. & Goodwin, T. W. (1969). In *Methods in Enzymology*. New York: Academic Press Inc. (in the Press).

Williams, R. J. H., Britton, G., Charlton, J. M. & Goodwin, T. W. (1967*a*). *Biochem. J.* **104**, 767.

Williams, R. J. H., Britton, G. & Goodwin, T. W. (1967*b*). *Biochem. J.* **105**, 99.