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EFFECT OF DIMETHYL SULFOXIDE (DMSO) ON THE BIOSYNTHESIS OF CAROTENOIDS IN DETACHED TOMATOES

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Abstract—β-Zeacarotene was isolated from the tomato fruit. Treatment with DMSO resulted in reduction of the total carotenoid content of the ripening fruit. The synthesis of phytoene, phytofluene, ζ-carotene, and lycopene was inhibited. The levels of β-zeacarotene, γ-carotene, and β-carotene remained essentially unchanged. Two possible modes of action of DMSO are discussed.

INTRODUCTION

PORTER AND LINCOLN[2] proposed a scheme for the biosynthesis of carotenoids based upon the pigment distribution in various tomato hybrids. A number of workers have subsequently modified the scheme to conform to recent structural proofs as well as to transformations in carotenogenic systems other than the tomato.[3–6]

While it is generally accepted that lycopene and β-carotene arise from phytoene, the nature of the immediate precursors of γ- and β-carotene is in question. Porter and Anderson[4] proposed alternative pathways for the formation of β-carotene and γ-carotene through lycopene and β-zeacarotene. Goodwin[6] has recently reviewed the evidence for and against the cyclization of lycopene in various carotenoid-forming systems. The point(s) of cyclization in the tomato fruit seems the least clear.

Isolated tomato fruit plastids have been shown to convert lycopene into γ-, δ- and β-carotene.[7] In the high β-carotene strain of the tomato, β-carotene is formed at the expense of lycopene.[2, 8, 9] However, the additional β-carotene formation is inhibited by higher ripening temperatures.[10, 11] The biosynthetic origin of the β-carotene in this strain may be different from that found in the normal red tomato, where β-carotene formation is not affected by ripening at temperatures above 30°.[10–12]

The inhibition of lycopene formation in ripening tomatoes by high temperatures with no effect on β-carotene synthesis seems to preclude a direct biosynthetic relationship between

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[2] J. W. PORTER and R. E. LINCOLN, *Arch. Biochem.* **27**, 390 (1950).
[3] S. L. JENSEN, G. COHEN-BAZIRE, T. O. M. NAKAYAMA and R. Y. STANIER, *Biochim. Biophys. Acta* **29**, 477, (1958).
[4] J. W. PORTER and D. G. ANDERSON, *Arch. Biochem. Biophys.* **97**, 520 (1962).
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[6] T. W. GOODWIN, In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. GOODWIN), p. 143. Academic Press, New York (1965).
[7] L. W. WELLS, W. J. SCHELBLE and J. W. PORTER, *Fed. Proc.* **23**, 426 (1964).
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[9] M. L. TOMES, F. W. QUACKENBUSH and M. MCQUISTAN, *Genetics* **39**, 810 (1954).
[10] M. L. TOMES, *Botan. Gaz.* **124**, 180 (1963).
[11] M. L. TOMES, F. W. QUACKENBUSH and T. E. KARGL, *Botan. Gaz.* **117**, 248 (1956).
[12] T. W. GOODWIN and M. JAMIKORN, *Nature* **170**, 104 (1952).

the two pigments. If lycopene were the only precursor of β-carotene, a gross inhibition of lycopene synthesis would result in a similar reduction in β-carotene production. In addition, Tomes *et al.*[8] reported that the introduction of the apricot gene (*at*) into the high-beta strain inhibited β-carotene production. According to Tomes *et al.*[8] and Jenkins and Mackinney[13] the apricot gene inhibits the production of lycopene but not of β-carotene in the normal red tomato.

Results of studies with radioactive terpenoid precursors have not produced unequivocal evidence for the biosynthetic origin of β-carotene. Specific activities of lycopene and β-carotene synthesized by isolated tomato plastids indicated that β-carotene may be formed by a pathway not involving lycopene.[14] However, the carotenoids synthesized by whole tomato fruit and isolated plastids derived from certain tomato selections showed a decreasing specific activity with increasing unsaturation, suggesting that a precursor–product relationship may exist between lycopene and β-carotene.[14, 15]

Lycopene does not appear to be involved in the formation of β-carotene in diphenylamine-inhibited cultures of *Phycomyces blakesleeanus*[16] or β-ionone-inhibited cultures of *Rhodotorula glutinis*.[5] It is also clear that γ-carotene is not derived from lycopene in the fungus *Rhizophlyctis rosea*.[22]

β-Zeacarotene has been proposed as the intermediate in the biosynthesis of β-carotene independent of lycopene.[4] It has been isolated from yellow corn, mold, yeast, and more recently in the flavedo of the fruit of a trigeneric Citrus hybrid Sinton citrangequat.[5, 17–20] The "unidentified carotene II" fraction isolated from tomato fruit by Porter and Zscheile[21] appears to be β-zeacarotene[4] but so far its identity has not been verified.

The objective of this study was to determine the effect of various inhibitors on the biosynthesis of tomato carotenoids during ripening. One of the agents, dimethyl sulfoxide (DMSO), was found to alter the pigmentation of detached, ripening tomatoes and its effect was studied further.

RESULTS AND DISCUSSION

β-Zeacarotene was isolated from tomato fruits. It was identified by its absorption spectrum and its relative position on magnesium oxide (MgO) columns.[5, 18] The equilibrium mixture obtained after iodine catalysis of synthetic β-zeacarotene and the tomato pigments were identical.[18]

Another pigment with maxima at 468, 442·5, 420, and 336 nm was isolated from a band chromatographing below β-carotene on the MgO columns. The pigment was tentatively identified as α-carotene. However, the pigment was subsequently shown to yield a different spectral curve after iodine catalysis from the α-carotene crystallized from carrot roots. The pigment formation was not inhibited by DMSO and thus may be related to the cyclic carotenes (Table 1).

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TABLE 1. EFFECT OF DMSO ON THE CAROTENOID CONTENT OF RIPENING, DETACHED TOMATO FRUIT*

	μg/g dry weight Days at 27°					
	0	4	6	8	10	12
Phytoene	0 0	0 0	21·4 0	40·1 3·5	24·3 2·3	22·9 7·5
Phytofluene	Trace Trace	4·7 1·8	11·2 4·4	21·8 4·2	39·5 7·7	45·0 4·4
ζ-Carotene	0 0	·4 0	·4 0	2·0 0	2·4 0	4·6 ·3
Lycopene	0 0	74·6 15·0	134·1 66·0	358·2 71·4	473·1 150·8	526·1 172·7
β-Zeacarotene	1·0 ·4	1·2 ·8	·5 ·4	1·0 ·7	·9 ·6	1·4 1·2
γ-Carotene	0 0	1·5 1·3	3·1 1·5	5·6 3·3	4·9 4·1	6·8 5·4
β-Carotene	13·1 10·2	22·3 15·4	34·1 25·5	33·2 32·2	35·4 45·5	49·9 52·5
α-Carotene†	2·5 ·9	2·6 4·8	8·9 4·4	7·6 5·4	10·2 12·0	11·2 8·8
Total	16·6 11·5	107·5 39·1	213·7 102·2	469·5 120·7	590·7 223·0	667·9 252·8

* The top figures in each category represent carotenoid content of the control while the bottom figures represent carotenoid content of DMSO-treated fruit.

† The pigment called α-carotene has subsequently been shown not to be identical to a sample of α-carotene crystallized from carrot roots.

Neurosporene was not detected in any analysis in this study. It is not present in detectable amount in red tomato strains. It has been isolated from a strain which produces large amounts of δ-carotene.[10]

DMSO inhibited red color formation in ripening tomatoes. Kinetic studies on pigment formation showed that DMSO inhibited the production of the acyclic carotenoids (phytoene, phytofluene, ζ-carotene, and lycopene). The synthesis of β-carotene and the other cyclic carotenoids, however, was not significantly affected (Table 1). The resulting, predominantly yellowish color of DMSO-treated fruits was, therefore, not due to increased β-carotene content nor to the stimulation of the production of any of the yellow-colored carotenes. It was the result of the inhibition of lycopene synthesis and the consequent reduction of the lycopene content of the ripe fruit. Treatment with DMSO also grossly reduced the total carotenoid content of the fruit since in untreated fruits lycopene and other acyclic compounds predominated.

The inhibitory effect of DMSO on carotenoid biosynthesis is similar to the effect of high temperatures on carotenoid synthesis in ripening tomatoes reported by Goodwin and Jamikorn.[12]

The presence of β-zeacarotene together with β-carotene and traces of phytofluene in

mature green tomato suggests that β-carotene is synthesized via β-zeacarotene and not by way of lycopene. However, the mere presence of β-zeacarotene in the system is not sufficient evidence for the exclusion of lycopene as the precursor of β-carotene. Though lycopene was not detected at the mature green stage, it is possible that there is a rapid turnover of lycopene→ γ-carotene→β-carotene so that not enough lycopene is present at this stage to be detectable. Treharne *et al.*[23] examined a number of corn mutants with blocks along the carotenoid biosynthetic pathway and found phytoene, phytofluene, ζ-carotene and neurosporene in one

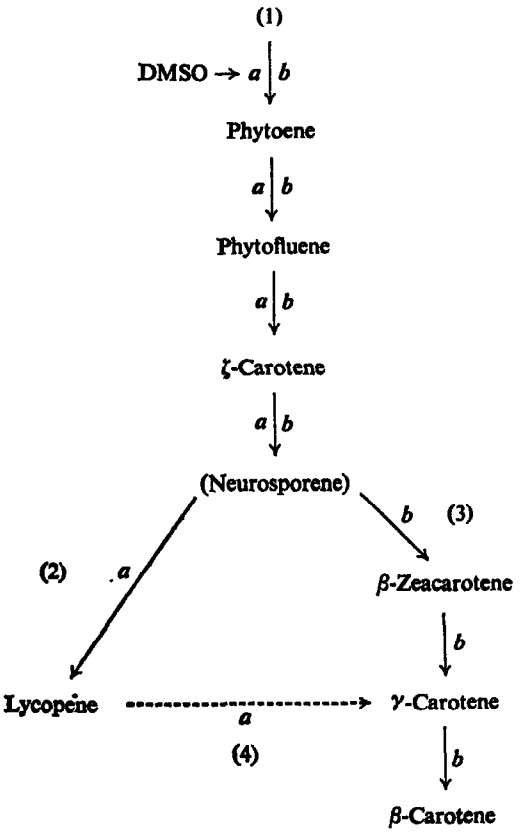

FIG. 1. THE SITE OF ACTION OF DMSO. *a* AND *b* REPRESENT TWO SEPARATE ENZYME SYSTEMS WHICH CAN BE LOCATED AT DIFFERENT SITES *in vivo*. 1, 2, 3, AND 4 REPRESENT POSSIBLE POINTS AT WHICH DMSO MIGHT ACT.

mutant; phytoene and phytofluene, and phytoene alone in others. These compounds do not accumulate in leaves of green, normal corn platns nor in etiolated, normal corn plants where β-carotene and lutein are the predominant carotenoids. These findings show that, although the more saturated carotenoids participate in the biosynthesis of the less saturated carotenoids, the former do not accumulate probably because of a rapid turnover of intermediates to products.

If one assumes a Porter–Anderson scheme for carotenoid formation (Fig. 1), and DMSO

[23] K. J. TREHARNE, E. I. MERCER and T. W. GOODWIN, *Phytochem.* **5**, 581 (1966).

blocked the pathway prior to phytoene (point 1), then theoretically the level of all the pigments should decrease. This assumption would seem logical, since the total concentration of pigments decreased. However, DMSO could not have acted only at point 1, because the level of all the cyclic carotenes remained essentially unchanged.

In order to explain the action of DMSO, one could also assume several combinations of inhibitions and activations at points 2, 3 and 4. It would seem improbable that such a complicated mechanism should exist for the effect of DMSO on the formation of carotenes in the tomato.

A more probable explanation of the inhibitory effect of DMSO on carotenoid synthesis assumes that carotenoids are synthesized by two separate enzyme systems which can be located at different sites *in vivo* (Fig. 1). The first site, *a*, would contain the series of enzymes for the phytoene→→lycopene synthesis and the second, *b*, the enzymes for the phytoene→→ β-zeacarotene→β-carotene synthesis. Site *a* would be inhibited by DMSO prior to phytoene; DMSO would not affect carotenoid synthesis in site *b*.

The preferential action of DMSO for one site might be explained on the basis of either of two possible modes of action:

(1) That lycopene is formed in site *a* during the transformation from the mature green to the full ripe stage while synthesis of the cyclic carotenoids in site *b* is initiated during the very early stage of fruit development continuing through the full ripe stage. If the enzyme system for the lycopene-forming site was formed during the transformation from the mature green to the full ripe stage, DMSO could have had an inhibitory effect on the synthesis of the enzyme system. The enzyme system for the cyclic carotenes would then be a "carry-over" from the system found in the green fruit. This argument is similar to the one advanced by Goodwin and Jamikorn[12] to explain the effect of high temperatures on carotenoid synthesis in ripening tomato.

(2) That a compartmentalization of similar enzymes exists in the tomato fruit. The mechanism of action of DMSO would then involve selective inhibition of different enzymes catalyzing the same reaction so that while the biosynthetic intermediates may be the same, the sites of synthesis are separated and isoenzymes may be involved. The mechanism for the regulation of terpenoid biosynthesis in developing seedlings proposed by Goodwin[24] involves a combination of compartmentalization of isoenzymes and selective permeability of the plastid membrane to mevalonic acid. This theory received substantial support with the isolation of extra- and intra-chloroplastidic mevalonic acid kinases by Rogers *et al.*[25] These enzymes were shown to possess different pH optima though catalyzing the same reaction. Thus, it is not unreasonable to assume that parallel pathways could exist within one system for the synthesis of various terpenoids.

If one assumes that isoenzymes are involved in the formation of phytoene, then one might also assume that one of the enzymes leading to the formation of lycopene might preferentially be inhibited by DMSO.

It has been suggested by Tomes[10] that the temperature sensitive step in the biosynthesis of carotenoids in ripening tomato is prior to phytoene. Since the mode of action of DMSO is similar to that of high temperature inhibition, the site of action of DMSO and temperature may be related.

The two-site hypothesis might explain the different specific activities obtained for lycopene

[24] T. W. Goodwin, In *Biosynthetic Pathways in Higher Plants* (Edited by J. B. Pridham and T. Swain), p. 57. Academic Press, New York (1965).
[25] L. J. Rogers, S. P. J. Shah and T. W. Goodwin, *Biochim. J.* **100**, 14c (1966).

and β-carotene synthesized from labeled precursors by whole tomato fruit and by tomato plastids.[14, 15]

The representation in Fig. 1 would be consistent with the statement by Tomes[10] that there are "two kinds of β-carotene". The DMSO data obtained in this investigation do not rule out the formation of β-zeacarotene, γ-carotene, and β-carotene in site *a* or lycopene in site *b* (Fig. 1). However, one would have to conclude on the basis of the evidence of the DMSO and temperature inhibition that the major pathway to β-carotene is through β-zeacarotene.

EXPERIMENTAL

Solvents. The solvents used—light petroleum (b.p. 30–50°), acetone, methanol, and diethyl ether—were all analytical reagent grade. They were distilled prior to use. Spectral grade pentane was used in the purification of crude phytoene.

Analytical reagent grade DMSO was used without prior distillation and was a gift of the Crown Zellerbach Corporation, Camas, Washington.

Chromatographic adsorbents. Sea Sorb 43 (Adsorptive Magnesia, MgO) and Hyflo Super-Cel were obtained from Fisher Scientific Co., Boston; the aluminum oxide, Woelm, neutral grade used in the purification of the various carotenoids was obtained from Alupharm Chemicals, New Orleans, Louisiana.

Other chemicals. Synthetic β-zeacarotene was a gift from Dr. O. Isler, F. Hoffman-LaRoche Ltd., Basle, Switzerland.

Fruits. Field-grown Summer Sunrise tomatoes were used in this study. The fruits were harvested 45–50 days from anthesis, that is, at the mature green stage.

Treatment with DMSO. The fruits were immersed in 10 per cent solution of DMSO for 30 min and transferred to the ripening room at 27–30°. Duplicate samples of four fruits each were removed from the ripening room at 2-day intervals and frozen at −17°. The frozen samples were held in the freezer until analyzed.

Pigment extraction and chromatography. Four fruits from each sampling date were removed from the freezer and homogenized in a high-speed Waring Blendor after thawing in a polyethylene bag at 30–40°.

The extraction procedure was similar to that described by Tomes.[11] The extract from a 50 g aliquot of the homogenate was saponified with 10% methanolic KOH on a steam table for 5–10 min. The process was carried out in the dark under a continuous stream of N_2. The unsaponifiable fraction was transferred to light petroleum by extraction with small amounts of acetone. Addition of water transferred the pigments to the epiphase. The ephiphase was washed free of alkali. Fifty millilitres of 85% methanol was then added to remove some of the xanthophylls.[11] The extract was washed free of methanol and dried over (Na_2SO_4) prior to chromatography.

The pigment extract was chromatographed in a tightly packed 22 × 200 mm column of MgO-Hyflo Super-Cel (1:2, w/w). The column was developed with 1% acetone–99% light petroleum (P.E.) followed by 5 and 10% acetone in P.E. The column was allowed to run dry after phytofluene had been eluted and the various bands were cut and eluted with acetone and methanol from the adsorbent.

The β-carotene zone which contained the β-zeacarotene was rechromatographed on a 20 mm column (12 mm i.d.) of alumina, activity grade I. The column was developed according to the procedure described by Simpson and Goodwin[19] for corn. β-Zeacarotene appeared as a tightly adsorbed band near the top of the column but often contaminated by an orange band. Rechromatography in alumina, activity grade III, resolved the mixture. β-Zeacarotene was eluted from the column upon the addition of 50 ml 1% ether followed by 5% ether.

Spectrophotometric analyses were made with a Cary 15 recording spectrophotometer.

Identification of the various carotenoids was made on the basis of their position on the columns and their absorption spectra.[26]

Iodine catalysis was accomplished by dissolving a small amount of iodine crystals in light petroleum. One or two drops of this solution were added to the spectrophotometer cell containing the pigment. The cell was subsequently exposed to light for 1–5 min.

Acknowledgements—This work was supported in part by National Science Foundation grant GB 3582.

[26] B. H. DAVIES, In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. GOODWIN), p. 489. Academic Press, New York (1965).