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BIOSYNTHESIS OF CAROTENOIDS IN THE TOMATO FRUIT

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Abstract—The effect of dimethyl sulfoxide (DMSO) on the biosynthesis of carotenoids in detached fruits of the "lutescent" tomato harvested at different stages of maturity was studied. DMSO inhibited the synthesis of both the acyclic and cyclic carotenoids in fruits treated prior to the lutescent stage. When more mature fruits were used the β-carotene content was not affected by DMSO while the levels of all other carotenoids examined significantly decreased. The pigment distribution in the fruit of the high-beta tomato genetic line was also studied. The high-beta fruit contained more β-zeacarotene and γ-carotene than the fruit of the normal red tomato "Summer Sunrise" of comparable ripeness. Treatment with DMSO inhibited the synthesis of both the acyclic and cyclic carotenoids in the high-beta tomato fruit.

INTRODUCTION

IT IS now generally accepted that the more saturated carotenoids are formed by dehydrogenation of the phytoene molecule. However, the point of cyclization is still ambiguous. The modified scheme proposed by Porter and Anderson[2] accommodated the evidence obtained from carotenogenic systems other than the tomato fruit, where lycopene does not appear to be an intermediate in the biogenesis of β-carotene.[3-6] This scheme proposes that β-carotene is formed by cyclization of both lycopene $\rightarrow$ γ-carotene $\rightarrow$ β-carotene, and neurosporene $\rightarrow$ β-zeacarotene $\rightarrow$ γ-carotene $\rightarrow$ β-carotene.

It was originally the result of genetic studies[7-9] of the inheritance of carotenoids in the tomato fruit which led Porter and Lincoln[10] to suggest that β-carotene is formed from lycopene. The conversion of lycopene to β-carotene by leaf chloroplasts,[11-14] tomato fruit plastids,[12-14] and a soluble extract of tomato fruit plastids[13] has been reported.

In the high-beta selection described by Porter and Lincoln,[10] the loss of lycopene was accompanied by the accumulation of a large amount of β-carotene in the fruit. The implication was that the β-carotene was derived from lycopene.[7] However, Tomes[15] and Tomes

[1] Contribution number 1331 of the Rhode Island Agricultural Experiment Station.
[2] J. W. PORTER and D. G. ANDERSON, *Arch. Biochem. Biophys.* **97**, 520 (1962).
[3] B. H. DAVIES, *Biochem. J.* **80**, 48P (1961).
[4] B. H. DAVIES, J. VILLOUTREIX, R. J. H. WILLIAMS and T. W. GOODWIN, *Biochem. J.* **89**, 96P (1963).
[5] T. W. GOODWIN, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 143, Academic Press, New York (1965).
[6] K. L. SIMPSON, T. O. M. NAKAYAMA and C. O. CHICHESTER, *J. Bacteriol.* **88**, 1688 (1964).
[7] G. W. KOHLER, R. E. LINCOLN, J. W. PORTER, F. P. ZSCHEILE, R. M. CALDWELL, R. H. HARPER and W. SILVER, *Bot. Gaz.* **109**, 219 (1947).
[8] G. MACKINNEY and J. A. JENKINS, *Proc. Nat. Acad. Sci. U.S.* **35**, 284 (1949).
[9] R. E. LINCOLN and J. W. PORTER, *Genetics* **35**, 206 (1950).
[10] J. W. PORTER and R. E. LINCOLN, *Arch. Biochem. Biophys.* **27**, 390 (1950).
[11] K. DECKER and H. UEHLEKE, *Hoppe-Seyl. Z. physiol. Chem.* **323**, 61 (1961).
[12] L. W. WELLS, W. J. SCHELBLE and J. W. PORTER, *Fed. Proc.* **23**, 426 (1964).
[13] S. KUSHWAHA, C. SUBBARAYAN, D. A. BEELER and J. W. PORTER, *J. Biol. Chem.* **244**, 3635 (1969).
[14] H. M. HILL and L. J. ROGERS, *Biochem. J.* **113**, 31P (1969).
[15] M. L. TOMES, *Bot. Gaz.* **124**, 180 (1963).

et al.[16] found that the β-carotene in the high-beta tomato is inhibited by ripening at temperatures above 30°. In the normal red parent as well as in other normal red lines[15-17] the β-carotene fraction is not sensitive to high temperature. Tomes[15] suggested that there may be two types of β-carotene in the tomato fruit—one type being synthesized through lycopene, which is sensitive to high temperature, the other through some pathway not involving lycopene which is not affected by high temperature.

It is also possible to inhibit lycopene synthesis in ripening fruit of the normal red tomato without affecting β-carotene formation by means of such diverse agents as irradiation[18] and treatment with dimethyl sulfoxide (DMSO).[19] The selective inhibitory effect of DMSO on the acyclic carotenoids of ripening normal red tomato[19] was explained on the basis of two possible modes of action: 1. That a compartmentalization of similar enzymes exists in the tomato fruit and that DMSO affects a key enzyme but not its isoenzyme. 2. That the enzyme system for the cyclic carotenes is formed early in the development of the fruit, whereas that of the acyclic carotenes is formed later and that DMSO in some way inhibits the latter system.

In order to test the second hypothesis an accurate determination of fruit maturity would be necessary. Critical determinations of the state of maturity of the green "Summer Sunrise" tomato are difficult. The "mature green" fruit of the "lutescent" tomato, however, is nearly devoid of chlorophyll so that carotene synthesis can be followed visually. The "lutescent" fruit normally goes from white to yellow to red.

If the additional level of β-carotene in the high-beta tomato is formed by cyclization of neurosporene → β-zeacarotene → γ-carotene, one might expect to find increased levels of β-zeacarotene and γ-carotene. The γ-carotene content of the tomato fruit has been found to increase in the presence of the BB gene.[15,20] β-Zeacarotene has only been isolated from the normal red tomato[19] and has not been reported in the high-beta tomato.

The objectives of this study were 1. to determine the effect of DMSO on carotenoid synthesis in the "lutescent" tomato fruit treated at different stages of maturity, 2. to determine the effect of DMSO on carotenoid synthesis in detached, mature green, high-beta tomato fruit, and 3. to attempt to delineate the biosynthetic pathway for β-carotene in the tomato fruit.

RESULTS AND DISCUSSION

The tomato fruits were harvested and separated into four stages of maturity. The following criteria were used. Stage 1: white, greenish around the stem-end; Stage 2: primarily white, slightly lutescent around the stem-end; Stage 3: lutescent, slight internal pink coloration; Stage 4: full yellow, externally visible red coloration at the blossom end and throughout the center of the fruit.

Preliminary ripening experiments using detached Stage 1 fruits resulted in highly variable pigmentation which was sufficiently great to preclude the use of Stage 1 fruits in this study. Stage 4 fruits showed less difference in color when compared with the control than Stages 2 and 3 after treatment with DMSO.

The kinetics of carotenoid synthesis in detached fruits of the "lutescent" tomato are shown in Tables 1 and 2. The formation of β-carotene appears complete after 3 days in fruits

[16] M. L. TOMES, F. W. QUACKENBUSH and T. E. KARGL, *Bot. Gaz.* **117**, 248 (1956).
[17] T. W. GOODWIN and M. JAMIKORN, *Nature* **170**, 104 (1952).
[18] E. E. BURNS and N. W. DESROSIER, *Food Tech.* **11**, 313 (1957).
[19] L. C. RAYMUNDO, A. E. GRIFFITHS and K. L. SIMPSON, *Phytochem.* **6**, 1527 (1967).
[20] M. L. TOMES, F. W. QUACKENBUSH and M. MCQUISTAN, *Genetics* **39**, 810 (1954).

that were harvested at Stage 3 (Table 1). Lycopene started to accumulate rapidly on the third day of ripening and reached the maximum level on the fifth day.

Treatment with DMSO inhibited the synthesis of the acyclic carotenoids phytoene, phytofluene, ζ-carotene and lycopene (Table 1). The level of inhibition is about the same

TABLE 1. EFFECT OF DMSO ON CAROTENOID SYNTHESIS IN RIPENING STAGE 3 FRUITS OF THE LUTESCENT TOMATO*

Polyene	μg/g dry wt. (days)					
	0	2	3	4	5	6
Phytoene	0·3	4·7	7·9	19·8	23·9	55·9
	0	0	2·4	0·5	4·0	4·0
Phytofluene	0·3	1·5	9·8	12·3	28·7	39·5
	Trace	0·7	1·5	1·9	2·5	2·2
ζ-Carotene	—	—	—	1·2	2·3	4·2
	—	—	—	Trace	0·3	Trace
Lycopene	4·9	62·4	118·8	235·1	402·0	334·5
	0	26·3	21·1	24·9	33·0	48·7
β-Zeacarotene	0·4	0·7	0·7	—†	1·4	—†
	0·2	0·9	0·9	0·6	0·7	0·9
γ-Carotene	0·3	2·8	5·4	8·1	9·7	7·7
	Trace	2·1	1·1	2·5	3·6	2·1
β-Carotene	27·7	49·5	60·4	59·6	64·7	59·6
	16·8	42·1	52·6	51·0	63·9	58·0
Total	33·9	121·6	203·0	336·1	532·7	501·4
	17·0	72·1	79·6	81·4	108·0	155·9

* The bottom figures represent the carotenoid content of DMSO-treated fruit; the top figures are for the control.

† β-Zeacarotene was observed but was lost on rechromatography.

order of magnitude as was reported for the normal red tomato.[19] Synthesis of the cyclic carotenes was not as drastically affected as the synthesis of the acyclic carotenoids. β-Carotene synthesis was not significantly different in the control and DMSO treatments. The β-zeacarotene concentrations were at the level of detectability. The γ-carotene content was consistently lower in DMSO-treated Stage 3 fruits than in the corresponding control fruits.

The lycopene content of fruits harvested at Stage 2 (Table 2) reached its maximum level on the ninth day of ripening. The maximum levels of β-zeacarotene and γ-carotene were attained on the eighth and ninth day, respectively. Phytoene, phytofluene and β-carotene were still accumulating at the end of the sampling period (11 days).

The formation of phytoene, phytofluene and lycopene was greatly inhibited by treatment of Stage 2 fruits with DMSO. In addition, the levels of β-zeacarotene and γ-carotene dropped markedly in DMSO-treated fruits. The β-carotene content was also reduced but the reduction was not as severe as that of the other carotenes.

Analysis of the carotenoid distribution in the high-beta tomato (Table 3) shows that the fruit contained not only more γ- and β-carotenes than the normal red (low-beta) tomato line but also more β-zeacarotene. Treatment with DMSO resulted in the reduction of the

TABLE 2. EFFECT OF DMSO ON CAROTENOID SYNTHESIS IN RIPENING STAGE 2 FRUITS OF THE LUTESCENT TOMATO*

Polyene	μg/g dry wt. (days)								
	0	2	3	4	5	6	8	9	11
Phytoene	0·2	0·6	4·6	12·3	17·3	22·7	51·6	69·2	97·5
	0·2	0·1	0·1	0·7	0·7	1·1	1·0	4·1	7·4
Phytofluene	0	Trace	1·9	3·7	14·8	19·9	41·0	44·4	56·1
	0	0	0	Trace	0·4	0·6	1·1	2·3	6·5
Lycopene	0	0·7	16·6	83·1	156·1	263·0	447·5	702·6	671·8
	0	0·1	0·4	1·0	0·8	1·1	6·3	22·6	92·2
β-Zeacarotene	0	Trace	Trace	2·0	1·9	3·1	4·7	3·8	3·6
	0	0	0	0·6	0·5	0·4	1·6	1·4	1·8
γ-Carotene	Trace	0·7	3·3	5·2	4·2	6·4	4·4	8·0	4·1
	Trace	0·2	0·2	0·4	0·5	0·7	0·8	1·2	0·9
β-Carotene	9·2	39·7	53·2	76·2	86·0	67·6	89·1	107·6	272·5
	10·4	24·6	33·5	37·7	46·6	49·5	87·7	107·1	127·4
Total	9·4	41·7	79·6	182·5	280·3	382·7	638·3	935·6	1105·6
	10·6	25·0	34·2	40·4	49·5	53·4	98·5	138·7	236·2

* The bottom figures represent carotenoid content of DMSO-treated fruit; the top figures are for the control.

TABLE 3. EFFECT OF DMSO ON THE BIOSYNTHESIS OF CAROTENOIDS IN THE HIGH-BETA TOMATO FRUIT*

Polyene	μg/g dry wt. (days)				
	0	6	8	12	12†
Phytoene	0	19·1	17·1	45·9	22·9
	0	3·1	10·7	21·4	7·5
Phytofluene	0	10·3	19·3	25·4	45·0
	0	1·6	7·5	10·8	4·4
Lycopene	—‡	—‡	—‡	—‡	526·1
					172·7
β-Zeacarotene	0·5	2·0	6·4	7·8	1·4
	1·4	0·6	2·5	1·4	1·2
γ-Carotene	0	8·6	10·5	11·8	6·8
	0	0·5	2·9	2·9	5·4
β-Carotene	26·3	360·1	549·6	1074·7	61·1§
	20·2	152·1	337·9	335·7	61·3
Total	26·8	400·1	602·9	1165·6	663·3
	21·6	157·9	361·5	372·2	252·5

* The bottom figures represent carotenoid content of DMSO-treated fruit, the top figures are for the control.

† Data from Raymundo *et al.*[19] on DMSO treatments on low-beta "Summer Sunrise" tomato strain added for comparison.

‡ Lycopene was not detected. The two reddish bands above γ-carotene on the (MgO) column were not lycopene as judged from their absorption spectra.

§ The values represent total of the β-carotene and "α-carotene" fractions. The latter fraction has been shown to be an isomer of β-carotene.

synthesis of both the acyclic and cyclic carotenoids in the high-beta fruit. Tomes[15] and Tomes *et al.*[20] reported previously that the high-beta tomato contains more γ- and β-carotenes than the normal red parent. β-Zeacarotene was not detected by these authors.

These results show that it is possible to partly inhibit the formation of β-carotene in the "lutescent" tomato if the detached fruits were exposed to DMSO prior to the appearance of the yellow pigmentation. The inhibition of β-carotene was not as extensive as the inhibition of lycopene and the other acyclic carotenes. These results are consistent with the proposal that two carotenogenic sites exist in the tomato fruit and that the formation of carotenes in a site once formed is not as susceptible to inhibition as in a developing site.

Thus, the system leading to β-carotene in the tomato fruit would seem to be formed at an earlier stage of maturity and is not as susceptible to inhibition as that system leading to lycopene. Dimethyl sulfoxide would appear to be involved in inhibiting the formation of the enzymatic system rather than inhibiting an existing system.

The results with the high-beta tomato would lead us to the conclusion expressed by Tomes[15] that there are two kinds of β-carotene in the tomato fruit. Our results are interpreted to show that the additional β-carotene is formed through a system developed at a later maturity and thus is susceptible to DMSO inhibition. In the "Summer Sunrise" tomato at approximately the same maturity as the high-beta tomato, the lycopene and not the β-carotene synthesis was inhibited by DMSO.

It is not possible from the DMSO data to exclude lycopene as a precursor of β-carotene. However, it would seem unlikely from these studies that lycopene is the major precursor of β-carotene. In the three tomato lines studied, β-zeacarotene and γ-carotene were inhibited to about the same extent. The finding of an increased level of β-zeacarotene in the high-beta tomato induced by the introduction of the BB, $mo_B mo_B$ genes would be consistent with the direct involvement of β-zeacarotene in the formation of γ- and β-carotenes.

Our conclusions are at variance with those reported by Kushwaha *et al.*,[13] who showed that the label from lycopene-15,15′-^{3}H is incorporated into γ-, β-, δ-, α- and neo-β-carotenes by spinach leaf chloroplasts and by soluble plastid extracts of the high-beta and high-delta tomato selections. Decker and Uehleke[11] showed that homogenates of the parenchymatous tissue of the tomato fruit could convert ^{14}C-lycopene to ^{14}C-β-carotene. More recently, Hill and Rogers[14] obtained a similar conversion with disrupted bean chloroplasts and disrupted tomato fruit plastids. Decker and Uehleke[11] also reported that the reaction lycopene → β-carotene was reversible.

It would seem that the conversion of lycopene to the cyclic carotenes in various plant tissue preparations has been well established. It would seem equally clear from the data on DMSO, irradiation and ripening temperature experiments that lycopene cannot be an obligate intermediate in the biosynthesis of β-carotene in the tomato fruit. Two possible conclusions could be drawn from the lycopene conversion studies that would be consistent with the data presented:

1. If the neurosporene → lycopene reaction were also reversible, the synthesis of β-carotene could have occurred through neurosporene and β-zeacarotene. The isolation of β-zeacarotene was not reported in any of the studies on the conversion of lycopene to the cyclic carotenes.

2. It is also possible that the cyclization enzymes require only a completed chromophore on the end to be closed and are not specific for the presence or absence of a double bond at the 7′,8′ position. Thus, lycopene could cyclize to γ-carotene and β-carotene; neurosporene would cyclize to β-zeacarotene. ζ-Carotene and β-zeacarotene would not be suitable sub-

strates for these enzymes. It would follow from this hypothesis that the ring-closure enzymes are not formed in the pathway leading to lycopene. However, if lycopene were added back to a plant tissue preparation, it might then be converted to γ-carotene and β-carotene.

Until the results of individual enzyme studies show otherwise, both schemes 6 and 7 of Williams *et al.*[21] would have to be considered in the interpretation of the data on the *in vitro* transformation of lycopene to other carotenes.

An analysis of the results of genetic studies by several workers[7-9,20,22,23] on the inheritance of the B^+/B, $mo_B{}^+/mo_B$ genes (Table 4) appears to provide additional evidence for the

TABLE 4. GENOTYPES OF THE LOW-BETA (NORMAL RED), INTERMEDIATE-BETA, AND HIGH-BETA TOMATO STRAINS[24]

Strain	Genotype*
Low-beta (normal red)	B^+B^+, $mo_B{}^+mo_B{}^+$
	B^+B^+, $mo_B{}^+mo_B$
	B^+B^+, mo_Bmo_B
Intermediate-beta	BB, $mo_B{}^+mo_B{}^+$
	B^+B, $mo_B{}^+mo_B$
High-beta	BB, mo_Bmo_B
	B^+B, mo_Bmo_B

* B is dominant over the B^+ allele; $mo_B{}^+$ is dominant over the mo_B allele.

existence of separate routes for lycopene and β-carotene in the tomato fruit. The rationale behind the suggested participation of lycopene in the synthesis of β-carotene was that since the total carotenoid content remained unchanged in the presence of the B allele in the high-beta genetic line, the additional β-carotene must have been derived from lycopene.[7,10] It might be assumed from this explanation that the B allele causes a shift in the equilibrium lycopene $\rightarrow$ γ-carotene $\rightarrow$ β-carotene towards β-carotene. However, Tomes[15] and Tomes *et al.*[16] reported that part of the β-carotene in the high-beta tomato, unlike that in the normal red line, can be inhibited by ripening at high temperature. Therefore, the origin of the additional β-carotene in the high-beta must have been different from that of the normal red tomato.

The inhibitory effect of the B^+/B, $mo_B{}^+/mo_B$ genes on lycopene formation may also be satisfactorily explained if one assumes that these genes control the balance between lycopene and β-carotene syntheses. In the presence of both dominant alleles B and $mo_B{}^+$, i.e. in the intermediate-beta line, both lycopene and β-carotene are synthesized in approximately equal amounts.[15] β-Carotene is produced only in large quantities, as in the high-beta line, when the interaction between the dominant B and the recessive mo_B alleles is expressed. Presumably, their products are required to produce an active repressor of the lycopene route, or alternatively, an effector substance for the β-carotene route. Finally, in the low-beta line, the β-carotene route is inoperative because the dominant B allele is absent.

[21] R. J. H. WILLIAMS, G. BRITTON and T. W. GOODWIN, *Biochem. J.* **105**, 99 (1967).
[22] M. L. TOMES, F. W. QUACKENBUSH, O. E. NELSON, JR., and B. NORTH, *Genetics* **38**, 117 (1953).
[23] M. L. TOMES, F. W. QUACKENBUSH and T. E. KARGL, *Botan. Gaz.* **119**, 250 (1958).
[24] J. T. O. KIRK and R. A. E. TILNEY-BASSETT, in *The Plastids, Their Chemistry, Structure, Growth, and Inheritance*, p. 344, W. H. Freeman and Co., San Francisco (1967).

The inhibition of β-zeacarotene and γ- and β-carotene syntheses in mature green, high-beta tomato fruit (Table 3) by treatment with DMSO supports this interpretation. It is the synthesis of the additional levels of these carotenes induced by the presence of the B and mo_B alleles that is blocked by DMSO. In the high-beta tomato, therefore, there exist two separate pathways for β-carotene. One pathway is not sensitive to DMSO applied at the mature green stage. This is the "carry-over" pathway from the immature fruit. The second pathway, the "inducible" pathway, is the one that is selectively inhibited by DMSO. In the high-beta tomato fruit the inducible pathway consists primarily of the β-carotene route, in the low-beta (normal red) fruit it consists largely of the lycopene route.

The inducible pathway is presumably repressed in the immature tomato fruit. Derepression possibly occurs prior to the appearance of the first visible coloration on the green fruit.

EXPERIMENTAL

Solvents and chromatographic adsorbents. The solvents and chromatographic adsorbents used were prepared as described previously.[19]

Fruits. The fruits of the "lutescent" and the high-beta tomato genetic lines were harvested from field-grown plants. Seeds of the high-beta tomato were generously provided by Dr. M. L. Tomes, Purdue University, Lafayette, Indiana, U.S.A.

Treatment with DMSO. The fruits were immersed in a 10% solution of DMSO for 1 hr and ripened at 27–30°. Each sample consisted of five fruits for the "lutescent" and three fruits for the high-beta line. The fruits were frozen at −17° and held in the freezer until analyzed.

Pigment extraction and chromatography. The extraction and purification procedures reported previously[19] were modified as follows: The extract was washed free of acetone and then saponified with 200 ml of 15% methanolic KOH on a steam table for 15–20 min. Saponification was carried out in the dark under N_2. The pigments were transferred to light petroleum ether (P.E.) by the usual procedure[19] and washed free of alkali.

The pigment extract was dried (Na_2SO_4) prior to chromatography. The column was developed first with P.E. until the phytofluene band separated from the β- (or α-) carotene band. Then 1% ether–99% P.E. followed by 2% ether–P.E. were added to move the β-carotene band.

When the γ-carotene band separated from the lycopene band, 5% ether–P.E. was added. β-Zeacarotene separated from γ-carotene upon the addition of 2% acetone–P.E. Addition of 5% acetone–P.E. further resolved the various bands. The column was extruded after phytofluene was eluted and the various bands were cut and eluted from the adsorbent with acetone or ethanol in P.E.

The β-zeacarotene obtained from the (MgO) column did not need further purification. However, when it is contaminated with a substantial amount of a γ-carotene isomer, as indicated by the appearance of an additional peak at around 485 nm, the pigment was rechromatographed on alumina (basic), activity grade III. β-Zeacarotene was eluted from the column with 1% ether–P.E.

Pigments more tightly adsorbed than lycopene were removed by passing the lycopene in P.E. washed free of ethanol through alumina III (neutral). The contaminants remained on the alumina. When γ-carotene appeared contaminated as judged from its spectrum, the pigment was rechromatographed on alumina III (neutral). The γ-carotene band was eluted from the column with P.E.

The fraction containing the phytoene collected from the (MgO) column was purified by rechromatography on 15 g of alumina (neutral), activity grade I. Spectral grade pentane or hexane was used in the purification procedure. 5-ml fractions were collected with a fraction collector. 70 ml of the solvent were collected before 150 ml of 1% ether in pentane or hexane were added to develop the column. This was followed by 150 ml of 2% ether. Phytoene appeared between fractions 30 and 50.

Pigment identification. The various carotenoids were identified on the basis of their position on the (MgO) column and their absorption spectra.[25]

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