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THE BIOSYNTHESIS OF CAROTENOIDS IN
THE TOMATO FRUIT

BY

LEONCIO CONDEZ RAYMUNDO

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOLOGICAL SCIENCES
(FOOD AND RESOURCE CHEMISTRY)

UNIVERSITY OF RHODE ISLAND

1971

ABSTRACT

Detached fruits of the "lutescent", high-beta and ghost tomatoes were treated with either dimethyl sulfoxide (DMSO) or 2-(4-chlorophenylthio)-triethylamine hydrochloride (CPTA) to study the biosynthetic origin of β -carotene in the tomato fruit. When mature "lutescent" tomato fruits (Stage 3) were treated with DMSO the synthesis of the acyclic carotenoids phytoene, phytofluene, ζ -carotene and lycopene was inhibited; the synthesis of β -carotene and the other cyclic carotenes was not affected. In less mature fruits (Stage 2), however, DMSO treatment inhibited the synthesis of both acyclic and cyclic carotenoids.

The synthesis of all the carotenoid fractions in high-beta fruits treated with DMSO at the mature green stage was inhibited. The high-beta fruit was found to contain increased levels of β -zeacarotene. Its synthesis, along with that of γ - and β -carotenes, decreased in the presence of DMSO. It was concluded, therefore, that the β -carotene fraction formed during ripening of the high-beta fruit is probably formed by cyclization of neurosporene to β -zeacarotene and not by cyclization of lycopene. It was suggested that the $B^+B, mo_B^+ mo_B$ genes probably control the balance between lycopene and β -carotene syntheses via a repressor-effector molecule interaction.

Total carotenoid accumulation in ripened "lutescent" and high-beta tomato fruits was reduced by DMSO treatment. CPTA did not affect carotenogenesis in the ghost tomato mutant. The results suggest that CPTA can exert its influence only in an already existing pathway and will not overcome inhibition imposed by genetic mutation. In contrast, lycopene

content increased fifteen-fold in the high-beta fruit treated with CPTA. The increase in the lycopene concentration was accompanied by a general increase in the level of the other acyclic carotenoids in the fruit. The β -zeacarotene, γ -carotene and β -carotene contents of the fruit decreased concomitantly. Total carotenoid accumulation in the high-beta fruit was reduced by CPTA.

A re-evaluation of the results of earlier work on the genetics of the inheritance of tomato fruit color and tomato plastid structural studies in conjunction with the findings in this study indicate that (1) the additional level of β -carotene in the high-beta fruit may be formed mainly via the β -zeacarotene route, (2) the chloroplast β -carotene is probably produced through a biosynthetically independent pathway from the carotenes of the chromoplast, and (3) the additional β -carotene fraction synthesized during ripening of the normal red tomato genotype is probably formed by a "carry-over" pathway from the green fruit.

A contaminant of the β -carotene fraction of the Tangerine tomato fruit extract was isolated and identified as a poly-cis isomer of ζ -carotene. The ripening fruit contains 0.8 ug/g dry weight of the pigment. The possible role of the poly-cis ζ -carotene in the biosynthesis of poly-cis carotenes is discussed.

PREFACE

The thesis was written to conform with the format outlined by the Graduate School for the Manuscript Thesis Plan.

Part I of this thesis was published in *Phytochemistry*, volume 9, pages 1239-1245 (1970); part II has been submitted to *The Biochemical Journal*, London. The data on the effect of CPTA on Phycomyces were furnished by Dr. C. O. Chichester's laboratory but were included in the second manuscript of the thesis because of the similarity of the response of the two carotenogenic systems to the chemical inhibitor and because the manuscript was submitted in this form. The third manuscript has been accepted for publication in *Phytochemistry*.

ACKNOWLEDGEMENTS

I owe much of whatever significant contribution this investigation has made to the area of carotenoid research to my adviser, Dr. Kenneth L. Simpson. It was such a pleasure to work for him that the seemingly intolerable task of literally working in the dark turned out to be a very intriguing one. I consider it a very rare opportunity, indeed, to have conducted all my graduate research work under his direction. For these and for his faith in my ability, I wish to express my profound gratitude to him.

I would also like to thank Dr. Luke S. Albert for his comments on the thesis and for his excellent course in Advanced Plant Physiology which greatly influenced my deductive reasoning and rationalization. To Dr. Albert E. Griffiths, I wish to extend my sincere appreciation for his help in growing the experimental plants used throughout this investigation, and for reviewing the final draft of this thesis and the manuscripts. In some instances, I did not agree with his comments and I am largely responsible for all the errors which are found in the text. I would also like to thank Dr. C. O. Chichester for serving in my committee despite his unbelievably busy schedule.

I am also personally indebted to Drs. Alberto and Ascunia Feliciano and their daughter Eva, for their patience, kindness and understanding, listening to my countless miseries and frustrations.

To my parents, Leoncio Bartolome and Elpidia Condez Raymundo.

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INTRODUCTION

Carotenoids are widely distributed in both the plant and the animal kingdoms. However, only plants are believed capable of synthesizing carotenoids de novo. Since carotenoids are precursors of Vitamin A required by animals for their growth and survival, animals rely on plants to fulfill their requirement for this vitamin. Plants, on the other hand, synthesize carotenoids primarily to protect their photosynthetic apparatus against photoinduced destruction catalyzed by their own chlorophyll or other light-absorbing compounds in the presence of oxygen. Only those molecules which have β -ionone ring possess provitamin A activity. Because the β -carotene molecule contains two such rings, it has theoretically the highest provitamin A activity among the naturally occurring carotenoids. β -Carotene is present in all photosynthetic tissues. Its role in these tissues, however, has not been clearly defined.

On ripening large amounts of carotenoids accumulate in the tomato fruit, in addition to the β -carotene and its oxygenated derivatives found in the immature green fruit. The kind and amount of carotenoid pigment synthesized during the transformation is largely dependent on the particular genotype of the plant with respect to fruit color. Temperature and light exert the most profound effects on carotenoid distribution in the tomato fruit among the various environmental factors affecting plant growth.

The more unsaturated carotenoids are formed by the sequential desaturation of phytoene. This compound is colorless because it has only three conjugated carbon-to-carbon double bonds. As the chromophore is

extended the molecule absorbs light at proportionately longer wavelengths. The absorbance falls in the visible region when seven double bonds are in conjugation. Thus the polyene is colored. Theoretically, the acyclic carotenoid molecule can have fifteen carbon-to-carbon double bonds in conjugation. Such a molecule will absorb light in the green region of the spectrum and therefore will impart a purple color.

The color intensity of the pigment is reduced by bending of the chromophore which is caused by either trans $\rightarrow$ cis rearrangement or by cyclization of the molecule. Spectroscopically, the absorption maxima are shifted toward the shorter wavelength. The absorption spectrum shows a loss of fine structure in the fundamental region with the concurrent appearance of cis-peak(s) in the near-ultraviolet region (Zechmeister, 1962).

The carotenoid molecule is extremely sensitive to trans $\rightarrow$ cis rearrangement because of the multiconjugated system. A large number of geometric isomers is, therefore, possible. Of the eleven conjugated double bonds in the lycopene molecule, however, only seven can readily undergo such rearrangement (Zechmeister, 1962). These are the methylated double bonds and the central double bond. Steric hindrance created by the overlapping of the neighboring hydrogen and methyl groups in the other positions reduces the probability of trans $\rightarrow$ cis rearrangement in the unmethylated positions (Pauling, 1939). Consequently, when an all-trans carotenoid is subjected to stereoisomerization, the formation of the unhindered cis isomers will predominate over that of the hindered forms.

The more unsaturated carotenoids are formed from phytoene. The immediate precursor for the cyclic carotenes, however, is still debatable. Structurally, lycopene is the most closely related acyclic carotenoid to the cyclic carotenes. However, high temperature and a number of chemicals can inhibit lycopene synthesis in the tomato fruit preferentially without affecting β -carotene synthesis. A precursor-product relationship between the two pigments is therefore considered highly unlikely. To circumvent the objection, an alternate pathway to the cyclic carotenes was proposed (Porter and Anderson, 1962) which does not involve lycopene. In this scheme, cyclization starts at neurosporene with β -zeacarotene as the intermediate.

It is the purpose of this investigation to attempt to delineate the biosynthetic pathway for β -carotene in the tomato fruit using the chemical inhibitors dimethyl sulfoxide (DMSO) and 2-(4-chlorophenylthio)-triethylamine hydrochloride (CPTA).

PART I. BIOSYNTHESIS OF CAROTENOIDS IN THE TOMATO FRUIT

BIOSYNTHESIS OF CAROTENOIDS IN THE TOMATO FRUIT

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Abstract--The effect of 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. The possible role of the $B^+/B, mo_{B^+}/mo_B$ genes is discussed.

INTRODUCTION

It is now generally accepted that the more unsaturated carotenoids are formed by dehydrogenation of the phytoene molecule. However, the point of cyclization is still ambiguous. The modified scheme proposed by Porter and Anderson² 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

¹Contribution number 1331 of the Rhode Island Agricultural Experiment Station.

²J. W. Porter and D. G. Anderson, Arch. Biochem. Biophys. **97**, 520 (1962).

³B. H. Davies, Biochem. J. **80**, 48p (1961).

⁴B. H. Davies, J. Villoutreix, R.J.H. Williams and T. W. Goodwin, Biochem. J. **89**, 96p (1963).

⁵T. W. Goodwin, Chemistry and Biochemistry of Plant Pigments, (Edited by T. W. Goodwin), p. 143. Academic Press, New York (1965).

⁶K. L. Simpson, T.O.M. Nakayama and C. O. Chichester, J. Bacteriol. **88**, 1688 (1964).

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 studies⁷⁻⁹ of the inheritance of carotenoids in the tomato fruit which led Porter and Lincoln¹⁰ 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 plastids¹³ 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, Tomes¹⁵ and Tomes 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 lines¹⁵⁻¹⁷ the β -carotene fraction is not sensitive to high tempera-

⁷G. 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).

⁸G. Mackinney and J. A. Jenkins, Proc. Nat. Acad. Sci. U.S. **35**, 284 (1949).

⁹R. E. Lincoln and J. W. Porter, Genetics **35**, 206 (1950).

¹⁰J. W. Porter and R. E. Lincoln, Arch. Biochem. Biophys. **27**, 390 (1950).

¹¹K. Decker and H. Uehleke, Hoppe-Seyl. Z. physiol. Chem. **323**, 61 (1961).

¹²L. W. Wells, W. J. Schelble and J. W. Porter, Fed. Proc. **23**, 426 (1964).

¹³S. Kushwaha, C. Subbarayan, D. A. Beeler and J. W. Porter, J. Biol. Chem. **244**, 3635 (1969).

¹⁴H. M. Hill and L. J. Rogers, Biochem. J. **113**, 31p (1969).

¹⁵M. L. Tomes, Bot. Gaz. **124**, 180 (1963).

¹⁶M. L. Tomes, F. W. Quackenbush and T. E. Kargl, Bot. Gaz. **117**, 248 (1956).

¹⁷T. W. Goodwin and M. Jamikorn, Nature **170**, 104 (1952).

ture. Tomes¹⁵ 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 irradiation¹⁸ and treatment with dimethyl sulfoxide (DMSO).¹⁹ The selective inhibitory effect of DMSO on the acyclic carotenoids of ripening normal red tomato¹⁹ 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 $\rightarrow \beta$ -zeacarotene $\rightarrow \gamma$ -carotene,

¹⁸E. E. Burns and N. W. Desrosier, Food Tech. 11, 313 (1957).

¹⁹L. C. Raymundo, A. E. Griffiths and K. L. Simpson, Phytochem. 6, 1527 (1967).

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 tomato¹⁹ 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.

²⁰M. L. Tomes, F. W. Quackenbush and M. McQuistan, Genetics 39, 810 (1954).

RESULTS AND DISCUSSION

The tomato fruits were harvested and separated into 4 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 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 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

Table I. 1. Effect of DMSO on carotenoid synthesis in ripening Stage 3 fruits of the "lutescent" tomato.*

POLYENE	$\mu\text{g/g}$ dry weight Days					
	0	2	3	4	5	6
Phytoene	$\frac{0.3}{0}$	$\frac{4.7}{0}$	$\frac{7.9}{2.4}$	$\frac{19.8}{0.5}$	$\frac{23.9}{4.0}$	$\frac{55.9}{4.0}$
Phytofluene	$\frac{0.3}{\text{Trace}}$	$\frac{1.5}{0.7}$	$\frac{9.8}{1.5}$	$\frac{12.3}{1.9}$	$\frac{28.7}{2.5}$	$\frac{39.5}{2.2}$
ζ -Carotene	$\frac{--}{--}$	$\frac{--}{--}$	$\frac{--}{--}$	$\frac{1.22}{\text{Trace}}$	$\frac{2.3}{0.3}$	$\frac{4.2}{\text{Trace}}$
Lycopene	$\frac{4.9}{0}$	$\frac{62.4}{26.3}$	$\frac{118.8}{21.1}$	$\frac{235.1}{24.9}$	$\frac{402.0}{33.0}$	$\frac{334.5}{48.7}$
β -Zeacarotene	$\frac{0.4}{0.2}$	$\frac{0.7}{0.9}$	$\frac{0.7}{0.9}$	$\frac{--+}{0.6}$	$\frac{1.4}{0.7}$	$\frac{--+}{0.9}$
γ -Carotene	$\frac{0.3}{\text{Trace}}$	$\frac{2.8}{2.1}$	$\frac{5.4}{1.1}$	$\frac{8.1}{2.5}$	$\frac{9.7}{3.6}$	$\frac{7.7}{2.1}$
β -Carotene	$\frac{27.7}{16.8}$	$\frac{49.5}{42.1}$	$\frac{60.4}{52.6}$	$\frac{59.6}{51.0}$	$\frac{64.7}{63.9}$	$\frac{59.6}{58.0}$
Total	$\frac{33.9}{16.4}$	$\frac{121.6}{72.1}$	$\frac{203.0}{79.6}$	$\frac{336.1}{81.4}$	$\frac{532.7}{108.0}$	$\frac{501.4}{155.9}$

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

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

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 eight 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 synthesis of both the acyclic and cyclic carotenoids in the high-beta fruit. ¹⁵Tomes and ²⁰Tomes et al. 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

Table I. 2. Effect of DMSO on carotenoid synthesis in ripening Stage 2 fruits of the "lutescent" tomato.*

	$\mu\text{g/g}$ dry weight								
	Days								
	0	2	3	4	5	6	8	9	11
Phytoene	$\frac{0.2}{0.2}$	$\frac{0.6}{0.1}$	$\frac{4.6}{0.1}$	$\frac{12.3}{0.7}$	$\frac{17.3}{0.7}$	$\frac{22.7}{1.1}$	$\frac{51.6}{1.0}$	$\frac{69.2}{4.1}$	$\frac{97.5}{7.4}$
Phytofluene	$\frac{0}{0}$	$\frac{\text{Trace}}{0}$	$\frac{1.9}{0}$	$\frac{3.7}{\text{Trace}}$	$\frac{14.8}{0.4}$	$\frac{19.9}{0.6}$	$\frac{41.0}{1.1}$	$\frac{44.4}{2.3}$	$\frac{56.1}{6.5}$
Lycopene	$\frac{0}{0}$	$\frac{0.7}{0.1}$	$\frac{16.6}{0.4}$	$\frac{83.1}{1.0}$	$\frac{156.1}{0.8}$	$\frac{263.0}{1.1}$	$\frac{447.5}{6.3}$	$\frac{702.6}{22.6}$	$\frac{671.8}{92.2}$
β -Zeacarotene	$\frac{0}{0}$	$\frac{\text{Trace}}{0}$	$\frac{\text{Trace}}{0}$	$\frac{2.0}{0.6}$	$\frac{1.9}{0.5}$	$\frac{3.1}{0.4}$	$\frac{4.7}{1.6}$	$\frac{3.8}{1.4}$	$\frac{3.6}{1.8}$
γ -Carotene	$\frac{\text{Trace}}{\text{Trace}}$	$\frac{0.7}{0.2}$	$\frac{3.3}{0.2}$	$\frac{5.2}{0.4}$	$\frac{4.2}{0.5}$	$\frac{6.4}{0.7}$	$\frac{4.4}{0.8}$	$\frac{8.0}{1.2}$	$\frac{4.1}{0.9}$
β -Carotene	$\frac{9.2}{10.4}$	$\frac{39.7}{24.6}$	$\frac{53.2}{33.5}$	$\frac{76.2}{37.7}$	$\frac{86.0}{46.6}$	$\frac{67.6}{49.5}$	$\frac{89.1}{87.7}$	$\frac{107.6}{107.1}$	$\frac{272.5}{127.4}$
Total	$\frac{9.4}{10.6}$	$\frac{41.7}{25.0}$	$\frac{79.6}{34.2}$	$\frac{182.5}{40.4}$	$\frac{280.3}{49.5}$	$\frac{382.7}{53.4}$	$\frac{638.3}{98.5}$	$\frac{935.6}{138.7}$	$\frac{1105.6}{236.2}$

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

Table I. 3. Effect of DMSO on the biosynthesis of carotenoids in the high-beta tomato fruit.*

POLYENE	$\mu\text{g/g}$ dry weight DAYS				
	0	6	8	12	12**
Phytoene	$\frac{0}{0}$	$\frac{19.1}{3.1}$	$\frac{17.1}{10.7}$	$\frac{45.9}{21.4}$	$\frac{22.9}{7.5}$
Phytofluene	$\frac{0}{0}$	$\frac{10.3}{1.6}$	$\frac{19.3}{7.5}$	$\frac{25.4}{10.8}$	$\frac{45.0}{4.4}$
Lycopene	---***	---***	---***	---***	$\frac{526.1}{172.7}$
β -Zeacarotene	$\frac{0.5}{1.4}$	$\frac{2.0}{0.6}$	$\frac{6.4}{2.5}$	$\frac{7.8}{1.4}$	$\frac{1.4}{1.2}$
γ -Carotene	$\frac{0}{0}$	$\frac{8.6}{0.5}$	$\frac{10.5}{2.9}$	$\frac{11.8}{2.9}$	$\frac{6.8}{5.4}$
β -Carotene	$\frac{26.3}{20.2}$	$\frac{360.1}{152.1}$	$\frac{549.6}{337.9}$	$\frac{1074.7}{335.7}$	$\frac{61.1****}{61.3}$
TOTAL	$\frac{26.8}{20.7}$	$\frac{400.1}{157.9}$	$\frac{602.9}{351.5}$	$\frac{1165.6}{372.2}$	$\frac{663.3}{252.5}$

*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 in 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.

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 Tomes¹⁵ 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_Bmo_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'-³H 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 Uehleke¹¹ showed that homogenates of the parenchymatous tissue of the tomato fruit could convert ¹⁴C-lycopene to ¹⁴C- β -carotene. More recently, Hill and Rogers¹⁴ obtained a similar conversion with disrupted bean chloroplasts and disrupted tomato fruit plastids. Decker and Uehleke¹¹ also reported that the reaction lycopene $\rightarrow$ 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 $\rightarrow$ 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 substrates 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 $\underline{B}^+/\underline{B}$, $\underline{mo}_B^+/\underline{mo}_B$ genes (Table 4) appears to provide additional evidence for the 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 $\underline{B}$ 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 $\underline{B}$ allele causes a shift in the equilibrium $\text{lycopene} \rightarrow \gamma\text{-carotene} \rightarrow \beta\text{-carotene}$ towards β -carotene.

²¹R.J.H. Williams, G. Britton and T. W. Goodwin, Biochem. J. 105, 99 (1967).

²²M. L. Tomes, F. W. Quackenbush, O. E. Nelson, Jr. and B. North, Genetics 38, 117 (1953).

²³M. L. Tomes, F. W. Quackenbush and T. E. Kargl, Bot. Gaz. 119, 250 (1958).

Table I. 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

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

²⁴J.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).

However, Tomes¹⁵ 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 $\underline{B}^+/B, \underline{mo}_B^+ \underline{mo}_B$ 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 $\underline{B}$ and $\underline{mo}_B^+$, 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 $\underline{B}$ and the recessive $\underline{mo}_B$ 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 $\underline{B}$ allele is absent.

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 $\underline{B}$ and $\underline{mo}_B$ 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" path-

way 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 5 fruits for the "lutescent" and 3 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 previously¹⁹ 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 a continuous stream of nitrogen gas. The pigments were transferred to light petroleum ether (P.E.) by the usual procedure¹⁹ and washed free of alkali.

The pigment extract was dried over (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 addi-

tion 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 -Zearotene 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. Five ml fractions were collected with a fraction collector. Seventy 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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²⁵B. H. Davies, In Chemistry and Biochemistry of Plant Pigments (Edited by T. W. Goodwin), p. 489. Academic Press, New York (1965).

PART II. THE EFFECT OF 2-(4-CHLOROPHENYLTHIO)-TRIETHYLAMINE HYDRO-
CHLORIDE (CPTA) ON CAROTENE FORMATION IN PHYCOMYCES AND
DETACHED TOMATO FRUIT

THE EFFECT OF 2-(4-CHLOROPHENYLTHIO)-TRIETHYLAMINE HYDROCHLORIDE
(CPTA) ON CAROTENE FORMATION IN PHYCOMYCES AND
DETACHED TOMATO FRUIT

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Recently Coggins, Henning & Yokoyama (1970) reported that 2-(4-chloro-phenylthio)-triethylamine hydrochloride (CPTA) causes the accumulation of lycopene in a wide spectrum of carotenogenic systems, such as fruits, vegetables, or moulds that either do not produce lycopene or produce it in minor amounts. Lycopene accumulation has also been reported by Howes & Batra (1970), in Mycobacterium marinum treated with nicotine, and by Knypf (1969), in pumpkin cotyledons treated with Cycocel [(2-chloroethyl)-trimethylammonium chloride].

In view of the wide response to CPTA and its suggested usefulness for studying carotene cyclization, it is of interest (i) to determine the changes in the other carotenes present, and (ii) to see whether CPTA can induce lycopene synthesis in mutants which accumulate large amounts of phytoene. The present paper reports the results of the analysis of the pigmentation pattern for CPTA-treated Phycomyces blakesleeana and an albino mutant of this mould. The effect of CPTA on pigmentation in the fruit of the high-beta and ghost tomato mutants is also reported.

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EXPERIMENTAL METHODS

P. blakesleeanus NRRL 1554- was obtained from the Northern Utilization Research and Development Division, U.S.D.A., Peoria, Ill., U.S.A. The P. blakesleeanus mutant albino 10 was kindly supplied by Dr. M. Delbruck of the California Institute of Technology, Pasadena, Calif., U.S.A. The tomato seeds were the generous gifts of Dr. M. L. Tomes of Purdue University, Lafayette, Indiana, U.S.A. (high-beta) and Dr. C. M. Rick of the University of California, Davis, Calif., U.S.A. (ghost).

The moulds were grown in a controlled environmental shaker (Psychrotherm, New Brunswick Scientific, New Brunswick, N. J., U.S.A.). Conditions were as follows: speed of shaker, 150 rpm; temperature, 20°C; and illumination, fluorescent lamps giving a light intensity of 3000 lux at the liquid surface. The high-beta and ghost plants were grown in the field and glasshouse, respectively; the fruits were harvested at the breaker stage of maturity and immediately treated.

CPTA at a concentration of 25 ppm was added to the mould culture 26 hours after inoculation from a spore suspension (cf. Lee and Chichester, 1969). The moulds were cultured for an additional 3 days prior to harvest.

Tomato fruits were injected with either distilled water or CPTA solution (4300 ppm) and allowed to ripen at 25°C for 12 days. Fruits injected with distilled water exhibited no observable change in the pigmentation pattern. CPTA treated fruits, however, developed red areas

around the point of injection. The red areas were dissected from each of the 12 treated high-beta fruit, combined and analyzed. The yellow portions of the fruit served as the control. The ghost tomato had no red areas following injection of CPTA and thus the whole fruit (32 per treatment) was used for analysis.

The extraction, purification, identification, and estimation of the carotenoid pigments of moulds (Lee and Chichester, 1969; Simpson, Nakayama and Chichester, 1964) and in tomatoes (Raymundo, Griffiths and Simpson, 1967, 1970) have been previously described in detail.

RESULTS

The results of the CPTA treatment of Phycomyces and the tomatoes are summarized in Table 1. When P. blakesleeianus was grown in the presence of 25 ppm of CPTA for 3 days the total carotenoid content increased by about 1/3. There was generally a slight increase in the acyclic carotenes with a large increase in the level of lycopene. β -Carotene was found to decrease, but CPTA caused a significant increase in the level of γ -carotene.

γ -Carotene was not detected in the untreated mould. The spores and the mycelia of the R_1 mutant of P. blakesleeianus do contain a large amount of γ -carotene and lycopene (Meissner and Delbruck, 1969; Lee et al., personal communication). Howes and Batra (1970) were not able to detect γ -carotene in untreated cells of M. marinum. However, γ -carotene could be isolated from cells treated with suboptimal amounts of nicotine which had resulted in a reduction of β -carotene. Increasing the concentration of CPTA up to 200 ppm resulted in additional increases in the lycopene concentration. Higher concentrations of CPTA tended to be toxic. Coggins et al. (1970) reported similar stimulation of lycopene synthesis in P. blakesleeianus with 15 ppm CPTA. However, they did not report the changes in the other carotenes.

Coggins et al. (1970) reported also that they treated 4 strains of tomatoes that do not form lycopene and found that lycopene became the major pigment. We treated the high-beta tomato fruit with CPTA and also

found a very large increase in lycopene. Analysis of the total carotene content showed that the increase in lycopene is accompanied by a general increase in the other acyclic carotenes. The cyclic carotenes, β -zeacarotene and γ -carotene, decreased slightly; however, there is a very large decrease in the level of β -carotene. The net effect of CPTA was to decrease the total carotene accumulation. These results are consistent with those of dimethylsulfoxide (DMSO) treatment of normal red, "lutescent" and high-beta tomato fruits, which led Raymundo *et al.* (1967, 1970) to propose that the main route of β -carotene synthesis is through β -zeacarotene rather than lycopene.

The results with the albino 10 mould are of particular interest since polyene synthesis genetically is blocked after phytoene. Total polyene synthesis was increased by CPTA as in the wild type mould, thus showing that the chemical was still able to stimulate the channelling of precursors to the phytoene level.

The effect of CPTA treatment on the ghost tomato was negligible. There was a very slight increase in lycopene and decrease in β -carotene with the total carotenoids nearly the same. The major polyene, phytoene, showed no change. Thus, in the albino 10 mould and ghost tomato where large amounts of the carotene precursor phytoene are already present, lycopene synthesis was not stimulated, suggesting that CPTA exerts its influence only in an already existing pathway and will not remove a block induced by genetic mutation. On the other hand, CPTA could act by

channelling the precursors away from β -carotene to lycopene synthesis. This is supported by the data on the high-beta tomato where both pathways are presumably operating (Raymundo, Griffiths and Simpson, 1970), the BB,/mo_Bmo_B genes simply shifting the synthesis toward β -carotene formation.

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Table II. 1. Carotenoid distribution in Phycomyces blakesleeana and in detached tomato fruits treated with CPTA.*

Polyene	<u>Phycomyces blakesleeana</u>		Tomato	
	Wild Type	albino 10	High-Beta	Ghost
Phytoene	$\frac{65}{85}$	$\frac{977}{1298}$	$\frac{107}{162}$	$\frac{1168}{1169}$
Phytofluene	$\frac{11}{35}$	$\frac{\text{Trace}^+}{-}$	$\frac{46}{73}$	$\frac{122}{123}$
ζ -Carotene	$\frac{16}{21} =$		$\frac{8}{16}$	$\frac{18}{28}$
Neurosporene	$\frac{6}{4}$		-	-
Lycopene	$\frac{2}{102}$		$\frac{40}{672}$	$\frac{36}{47}$
β -Zeacarotene	$\frac{-}{\text{Trace}}$		$\frac{4}{2}$	-
γ -Carotene	$\frac{-}{58}$		$\frac{47}{35}$	-
β -Carotene	$\frac{393}{361}$		$\frac{1398}{360}$	$\frac{14}{5}$
Total	$\frac{498}{666}$	$\frac{977}{1298}$	$\frac{1650}{1291}$	$\frac{1358}{1372}$

*The top figure in each category represents the carotenoid content (in ug/g dry weight) of the control and the bottom figure represents the value for the corresponding treated material. Each figure is the average of duplicate determinations.

+Detected on the column when viewed with UV light.

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PART III. THE ISOLATION OF A POLY-CIS ζ -CAROTENE FROM THE TANGERINE TOMATO

THE ISOLATION OF A POLY-cis ζ -CAROTENE FROM
THE TANGERINE TOMATO¹

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Abstract--A contaminant of the β -carotene fraction of tangerine tomato fruit extract was isolated and identified as a poly-cis isomer of ζ -carotene. The ripening fruit contains 0.8 ug/g dry weight of the pigment. The possible role of poly-cis ζ -carotene in the biosynthesis of poly-cis carotenes is discussed.

INTRODUCTION

Tangerine tomato fruits contain as the characteristic pigments the poly-cis carotenes proneurosporene and prolycopene² and smaller amounts of cis isomers of phytofluene and ζ -carotene.³ Compared with ordinary tomatoes, the tangerine fruits have reduced levels of all-trans lycopene and β -carotene, but greatly increased amounts of the more saturated carotene precursors phytoene, phytofluene and ζ -carotene.

¹Contribution Number 1383 of the Rhode Island Agricultural Experiment Station.

²A. L. LeRosen and L. Zechmeister, J. Amer. Chem. Soc. 64, 1075 (1942).

³R. J. H. Williams, G. Britton, J. M. Charlton and T. W. Goodwin, Biochem. J. 104, 767 (1967).

The phytoene and phytofluene levels in the ripe tangerine tomato are surpassed only by the levels of these polyenes in the ghost tomato.^{4,5} The all-trans ζ -carotene content which has been reported to amount to 414.4 ug/g dry weight³ is the highest concentration of this pigment found in tomato fruits.^{6,7} The β -carotene content, on the other hand, is more typical of the values reported for green tomatoes.⁴

In the present paper we report the identification of a poly-cis isomer of ζ -carotene from extracts of tangerine tomatoes.

RESULTS AND DISCUSSION

A contaminant of the β -carotene fraction eluted from the MgO: Hyflo Super Cel column was isolated during the purification of β -carotene by TLC on silica gel F plates. The adsorption maxima of the yellow pigment were different from any of the carotenes whose fundamental band falls between the 370 and 420 nm region of the spectrum.

⁴G. Mackinney, C. M. Rick and J. A. Jenkins, Proc. Nat. Acad. Sci. U.S. 42, 404 (1956).

⁵M. L. Tones, Bot. Gaz. 124, 180 (1963).

⁶J. W. Porter and F. P. Zscheile, Arch. Biochem. 10, 537 (1946).

⁷J. W. Porter and R. E. Lincoln, Arch. Biochem. 27, 390 (1950).

The pigment exhibited λ_{max} at 414, 390 and 369 nm which shifted to 422, 397 and 377 nm on the addition of catalytic amounts of iodine (Fig. 1). The extinction of the fundamental region also increased concomitant with the bathochromic shift. The absorption of the iodine catalyzed equilibrium mixture is identical to the spectral curve of the stereoisomers obtained upon iodine catalysis of the all-trans ζ -carotene.

A strong bathochromic shift and an increase in the extinction at the fundamental region on iodine catalysis are characteristic properties exhibited by all poly-cis carotenoids.⁸ These data are consistent with the conclusion that the isolate is a poly-cis isomer of ζ -carotene.

In the ripening fruit the poly-cis ζ -carotene was found at a concentration of 0.8 ug/g dry weight which is about 0.4% of the total ζ -carotene content of 189.2 ug/g dry weight.

A comparison of the absorption maxima of the poly-cis ζ -carotene with the spectrum of some synthetic and natural isomers of ζ -carotene is given in Table 1.

⁸L. Zechmeister, cis-trans Isomeric Carotenoids, Vitamins A and Aryl-polyenes, Springer-Verlag: Vienna (1963).

Fig. III.1. Specific extinction curves of poly-cis ζ -carotene, in petroleum ether: (a) fresh solution; (b) mixture of stereoisomers after catalysis by iodine; and (c) mixture of stereoisomers after catalysis of all-trans ζ -carotene by iodine.

Table III. 1. λ_{max} of the poly-cis ζ -Carotene and Some Natural and Synthetic Isomers

Isomer	Source	max, nm				Reference	
all- <u>trans</u> ζ -Carotene	Carrot oil	425	400	378	(361)	9	
all- <u>trans</u> ζ -Carotene	Synthetic	425	401	380		10	
unsym. ζ -Carotene	Synthetic	420	395	375	(356)	9, 10	
unsym. ζ -Carotene (7,8,11,12-Tetra- hydrolycopene)	<u>Rhodospirillum rubrum</u>	418.5	394.5	374	(354)	11	
"neo A" ζ -Carotene	Carrot oil	419	396	375	(357)	296	285 9
<u>cis</u> -15- ζ -Carotene	Synthetic	422	398	378	(359)	296	286 9, 10
<u>cis</u> ζ -Carotene	Tangerine tomato	423	398	378			3
poly- <u>cis</u> ζ -Carotene	Tangerine tomato	414	390	369	(352)	296	285

(34)

⁹J. B. Davis, L. M. Jackman, P. T. Siddons and B. C. L. Weedon, J. Chem. Soc. (C), 2154 (1966).

¹⁰J. B. Davis, L. M. Jackman, P. T. Siddons and B. C. L. Weedon, Proc. Chem. Soc., 261 (1961).

¹¹B. H. Davies, Biochem. J. 116, 93 (1970).

The structure of poly-cis carotenoids has not been satisfactorily elucidated. Based on spectral studies of cis-trans carotenoids and related polyenes, Zechmeister⁸ suggested that polycopene has a symmetrical structure with cis configuration at the central double bond and 4 other sterically unhindered double bonds. The unhindered positions are the methylated carbon-to-carbon double bonds in the carotenoid molecule.¹² Introduction of hindered cis double bond (-s) results in the loss of fine structure in the fundamental region.^{8,13,14}

The absence of a strong cis-peak in the near ultraviolet region of the poly-cis ζ -carotene spectrum suggests that the molecule does not have a bent shape. The central mono-cis isomer, e.g. central mono-cis isomers of β -carotene and lycopene, has the strongest cis-peak because of its bent shape; conversely, the spectral curve of the all-trans isomer has a relatively flat, although slightly elevated cis-peak region.⁸

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¹³C. H. Eugster, C. F. Garbers and P. Karrer, Helv. Chim. Acta 36, 1378 (1953).

¹⁴O. von Isler, L. H. Chopard-dit-Jean, M. Montavon, R. Ruegg and P. Zeller, Helv. Chim. Acta 40, 1256 (1957).

In view of our present isolation of a poly-cis ζ -carotene, we suggest that the proposed pathway of Porter and Anderson¹⁵ be modified to include this new compound as an intermediate. The poly-cis ζ -carotene could be formed from the all-trans ζ -carotene. The existence of three poly-cis polyenes would lead one to suggest that poly-cis phytoene and phytofluene might also be formed in the system (Fig. 2). These compounds, however, have not been reported. If they were formed their poly-cis nature would be difficult to detect since most of the unhindered cis positions would be out of conjugation. Such a parallel pathway would be similar to one proposed by Malhotra, Britton and Goodwin¹⁷ for the biosynthesis of 1,2-dihydro carotenes from 1,2-dihydro phytoene in Rhodopseudomonas viridis.

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Fig. III,2. Possible pathways leading to the formation of polycopene.

PERMANENT RECORD

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75% COTTON FIBER CONTENT

PERMANENT RECORD

SOUTHWEST CALIFORNIA

EXPERIMENTAL

Materials--Field ripened Tangerine tomato fruits were used in this study. The seeds were a gift from Dr. M. L. Tomes of the Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana, U.S.A.

Pigment Extraction and Purification--Thirty-gram aliquots of the fruit were extracted with acetone-petroleum ether (P.E., b.p. 30-50⁰) in a Waring Blendor under a continuous stream of nitrogen gas. The extract was washed immediately with water to remove the acetone. The extract was either chromatographed after drying (Na_2SO_4) without prior saponification or saponified with 10% KOH in methanol (w/v). Saponification was accomplished by evaporating the P.E. in a flash evaporator to transfer the pigment extract into the methanolic solution. The extract was transferred to P.E. after 15 min. following the usual procedure,¹⁸ dried (Na_2SO_4) and chromatographed on a 22 x 360 mm column of MgO:Hyflo Super Cel (1:2, w/w). The chromatogram was developed with 2, 5 and finally 10% acetone in P.E. The column plug was extruded and the zone below the cis ζ -carotene band was cut together with the β -carotene band. The pigments were eluted from the adsorbent with acetone-P.E., evaporated to dryness and rechromatographed on a 12 x 150 column of alumina II.

¹⁸L. C. Raymundo, A. E. Griffiths and K. L. Simpson, *Phytochem.* 6, 1527 (1967).

The column was developed with increasing concentrations of diethyl ether in P.E. The poly-cis ζ -carotene was eluted from the column together with the β -carotene band with 5% ether. The eluate was evaporated to dryness and purified by TLC on precoated silica gel F₂₅₄ plates with P.E. as developing solvent. The yellow band adsorbed below the β -carotene band is the poly-cis ζ -carotene band. Alternatively, the eluate from the MgO: Hyflo Super Cel column was first purified by TLC on precoated silica gel F₂₅₄ plates to remove the β -carotene. The poly-cis ζ -carotene band was eluted from the adsorbent and rechromatographed on a short column of alumina II to remove a contaminating β -zeacarotene-like pigment.

Absorption Spectra--The absorption spectra of the carotenes were recorded from solutions in P.E. contained in a glass-covered quartz cell with a Cary 15 recording spectrophotometer. Quantitative estimation of the poly-cis ζ -carotene was made from the equilibrium mixture of stereoisomers after the poly-cis pigment was subjected to iodine catalysis using the $E_{1\text{cm}}^{1\%}$ value of 2270 for the all-trans ζ -carotene at 400 nm.¹⁹

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¹⁹B. H. Davies, In Chemistry and Biochemistry of Plant Pigments, (edited by T. W. Goodwin), p. 489, Academic Press: New York (1965).

APPENDIX

I. IN RETROSPECT

A. The Protective Function of Carotenoids Against Photosensitized Oxidation

Carotenoids are invariably the most widely distributed pigment in Nature (Goodwin, 1965a). They are present without exception in all photosynthetic tissues of prokaryotic and eukaryotic organisms. To explain the ubiquity of carotenoids, attempts have been made to define their function among carotenogenic organisms by correlating their presence to certain physiological and morphological responses (Burnett, 1965). So far, however, only their protective function against photosensitized oxidation has been thoroughly and unambiguously documented (Krinsky, 1968).

Griffiths, Sistrom, Cohen-Bazire and Stanier (1955) demonstrated the protective role of carotenoids in Rhodopseudomonas spheroides against photooxidation catalyzed by its own bacteriochlorophyll. Cells of a blue-green mutant devoid of colored carotenoids were killed when illuminated under aerobic condition but remained normal in the dark or at low light intensity, or when grown anaerobically in the presence of light. Similar observations have been made by Claes (1954) in Chlorella vulgaris in which mutants accumulating the more saturated carotenes, phytoene, phytofluene and ζ -carotene were killed when exposed to visible light in the presence of oxygen. Chance and Sager (1957) also reported the photodynamic killing of an analogous mutant of Chlamydomonas reinhardtii.

Photooxidative destruction of chlorophyll in the absence of colored carotenoids has also been reported in higher plants (Koski and Smith,

1951; Wallace and Schwarting, 1954; Smith, Durham and Wurster, 1959; Anderson and Robertson, 1960, 1963; and Walles, 1967). Walles (1965, 1966) described the structural changes in plastids of dark-grown albina and xantha mutants of sunflower which lead to bleaching upon illumination. He concluded that carotenoids do not influence the synthesis and organization of the prolamellar body in the developing plastid.

Unlike the wild type, the xantha mutant accumulates xanthophylls but not β -carotene while the albina mutant does not synthesize colored carotenoids (Habermann, 1960). Xantha does not bleach as rapidly as the albina mutant on illumination, implying that the xanthophylls offer some degree of protection against photosensitized oxidation and that β -carotene is necessary to maintain the structural integrity of the plastids formed in the dark. The grana and the chlorophyll incorporated into the grana are unstable and rapidly destroyed by light in the absence of β -carotene (Walles, 1967).

Faludi-Daniel and Lang (1964) described two mutants of corn, one of which accumulates ζ -carotene while the other accumulates lycopene. The chlorophyll formed by these mutants at low light intensities is destroyed on subsequent exposure to high light intensities. An analogous situation was observed in albescent corn mutant (Sander, Laber, Bell and Hamilton, 1968).

The protective function of carotenoids against lethal photosensitized oxidations by visible light has been reported in non-photosynthetic chromogenic bacteria (Mathews and Sistrom, 1959; Wright and Rilling, 1963;

Mathews, 1964; Mathews-Roth and Krinsky, 1970a,b,c) and in yeasts (Chichester and Maxwell, 1969). The photodynamic effect of visible light in these organisms is catalyzed by other light-absorbing compounds. A comprehensive review of the subject was made by Krinsky (1968).

Krinsky (1966, 1968) suggested a scheme for the deactivation of the excited chlorophyll-oxygen complex. It involves epoxidation of zeaxanthin to antheraxanthin which is a non-enzymatic process; regeneration of zeaxanthin is catalyzed by antheraxanthin de-epoxidase. Yamamoto, Nakayama and Chichester (1962) observed that violaxanthin can be converted to zeaxanthin via antheraxanthin anaerobically in light by leaf segments from spinach and lima beans. They concluded that the conversion is enzymic because a mild heat treatment can reduce the reaction. Dworkin (1959) did not observe bleaching of chlorophyll in Rhodopseudomonas spheroides at 1° although photokilling occurred, suggesting that part of the protective mechanism is enzymic. Carotenoids of pigmented Sarcina lutea offered lesser protection against photosensitization by toluidine blue in air at 4° than at 34° (Mathews, 1964).

Yamamoto and Chichester (1965) also reported the incorporation of O₂ into antheraxanthin in the dark following exposure of bean leaves to intense light.

The mechanism that has been suggested whereby carotenoids are able to quench the excited state of chlorophyll is the photoinduced isomerization of carotenoids. Rau and Zehender (1959) found that the fungus

Fusarium aquaeductum accumulated poly-cis lycopene II when aerated in light. By x-ray irradiation, Claes (1954, 1956, 1957) obtained a Chlorella mutant which produces prolycopene and proneurosporene when grown in the dark. On subsequent illumination of the dark-grown culture with blue light, the poly-cis isomers disappeared concomittant with the formation of all-trans lycopene and all-trans neurosporene. According to Claes and Nakayama (1959) cis $\rightarrow$ trans isomerization can also occur in red light both in vivo and in vitro provided that chlorophyll is present. Apparently, the chlorophyll acts as the photosensitizer. The photoinduced isomerization was inhibited by oxygen. Schroeder (1942) similarly reported the presence of prolycopene and pro- γ -carotene in the petals of the monkey flower, Mimulus longiflorus, at low light intensity; at high light intensity, only the corresponding all-trans isomers are produced.

Reviews of the mechanisms of sensitized photooxidation in biological systems have been published recently (Spikes and Straight, 1967; Foote, 1968).

The herbicidal property of 3-amino-1,2,3-triazole (Amitrole), 3,4-dichlorobenzyl methylcarbamate (Dichlormate) and 2,3,5-trichloro-4-pyridinol (Pyrictlor) has been attributed to the inhibition of β -carotene synthesis with the concurrent accumulation of ξ -carotene, phytofluene and phytoene (Burns, Buchanan and Carter, 1971). These carotenoids are not capable of protecting the chlorophyll from photooxidative destruction sensitized by the chlorophyll itself (Mathews-Roth and Krinsky, 1970).

The chloroplastidic β -carotene may be involved in some way in the protection of the plastids of developing tomato fruit from photosensitized oxidation. Zeaxanthin and its epoxides are derived from β -carotene in green tomato plant leaves (Glover and Redfearn, 1953). These oxygenated β -carotene derivatives may be involved in the deactivation of the chlorophyll-oxygen complex (Blass, Anderson and Calvin, 1959; Yamamoto, Nakayama and Chichester, 1962). Thus the chloroplastidic β -carotene may serve as a precursor for its oxygenated derivatives involved in photoprotection. The role of the carotenoid pigments synthesized during the ripening of the tomato fruit is still obscure.

B. The Genetics of the Inheritance of Chromoplast Carotenoids in the Tomato Fruit

The carotenoids in the chromoplasts exhibit a wider degree of qualitative and quantitative variability than do carotenoids in the chloroplasts. The greater mutability of chromoplasts compared to the latter organelles has never been satisfactorily explained, especially considering that chromoplasts may be derived from chloroplasts (Frey-Wyssling and Muhlethaler, 1965).

Most of the genes studied so far which determine tomato fruit color do not affect the amount and/or kind of carotenoids present in the leaves and green fruit. There are two exceptions: the high pigment gene, hp^+/hp , and the ghost gene, gh^+/gh . The recessive mutant allele, hp , increases both the chlorophyll content and the amount of the different carotenoids in the leaves and green fruit and in ripe fruits (Baker and Tomes, 1964).

The recessive mutant allele, gh, causes a drastic loss of chlorophyll and colored carotenoids in both leaves and fruits (Mackinney, Rick and Jenkins, 1956; Rick, Thompson and Brauer, 1959).

The genotypes of several tomato fruit color mutants are shown in Table 1.

The r^+/r gene affects the total amount of carotenoids in the ripe fruit (LeRosen, Went and Zechmeister, 1941; Jenkins and Mackinney, 1955). It was suggested (Mackinney, Rick and Jenkins, 1956) that the recessive allele, r, blocks the carotenoid pathway prior to phytoene since this polyene does not accumulate in the rr fruit.

The homozygous recessive allele, t, causes phytoene, phytofluene, ζ -carotene and neurosporene to accumulate in the fruit (Williams, Britton, Charlton and Goodwin, 1967). The most dramatic effect of the t^+/t gene, however, is the accumulation of the poly-cis isomers of lycopene and neurosporene, prolycopene and proneurosporene, in the tt genotype. The t^+/t gene may act by determining the spatial configuration of the carotene molecule (Zechmeister and Went, 1948), or it might control a dehydrogenase involved in the conversion of the poly-cis carotenoids to lycopene (Zechmeister, LeRosen, Went and Pauling, 1941; Mackinney and Jenkins, 1952; Porter and Anderson, 1967).

Jenkins and Mackinney (1955) discovered the apricot gene, at^+/at . The recessive allele, at, reduces the total carotenoid content in the fruit and at the same time suppresses lycopene synthesis but not that of

Table 1
Genotypes of Tomato Fruit Color Mutants

Color Variety	r^+/r	t^+/t	at^+/at	B/B^+	mo_B^+/mo_B	Del^+/Del	og^C/og^C	hp^+/hp	gh^+/gh	sh^+/sh
Red	+	+	+	B^+B^+	$mo_B^+mo_B^+$	+	+	+	+	+
Tangerine	+	tt	+	+	+	+	+	+	+	+
Apricot	+	+	atat	+	+	+	+	+	+	+
High-beta	+	+	+	BB	mo_Bmo_B	+	+	+	+	+
Intermediate-beta	+	+	+	BB	$mo_B^+mo_B^+$	+	+	+	+	+
Delta	+	+	+	+	+	$DelDel$	+	+	+	+
Crimson	+	+	+	+	+	+	og^Cog^C	+	+	+
High Pigment	+	+	+	+	+	+	+	hphp	+	+
Low Pigment	rr	+	+	+	+	+	+	+	+	+
Ghost	+	+	+	+	+	+	+	+	ghgh	+
Sherry	+	+	+	+	+	+	+	+	+	shsh

+indicates dominance except in the B^+/B and Del^+/Del genes where B^+ and Del^+ are the recessive alleles, respectively.

β -carotene. In the BB fruit, however, atat inhibits the synthesis of β -carotene (Tomes, Quackenbush and Kargl, 1958) which presumably was produced by cyclization of lycopene (Kohler, Lincoln, Porter, Zscheile, Caldwell, Harper and Silver, 1947; Lincoln and Porter, 1950; Porter and Lincoln, 1952).

The B^+/B gene determines whether the major carotenoid in the fruit is lycopene or β -carotene (Kohler et al., 1947; Lincoln and Porter, 1950). Its effect is modified by another set of independently inherited genes, mo_B^+/mo_B . The dominant allele, B , causes the accumulation of large amounts of β -carotene in the fruit; the recessive allele, B^+ , in the presence of the dominant allele, mo_B^+ , shifts the pathway to lycopene synthesis. However, when the dominant alleles B and mo_B^+ are together, the fruit produces approximately equal amounts of lycopene and β -carotene (Tomes, 1963). Tomes, Quackenbush and Kargl (1956) suggested that the function of the B allele is to mediate the formation of the β -ionone ring.

The Del^+/Del gene appears to control the formation of the α -ionone ring (Kargl, Quackenbush and Tomes, 1960). Fruits containing the dominant allele, Del , contain about 40% less carotenoids than the Del^+ tomato and the major carotenoid is δ -carotene.

Substitution of the B allele for B^+ in a $DelDel$ genotype results in the reduction of α - and δ -carotenes (Tomes, 1967); the substitution of either B or Del for B^+ or Del^+ , respectively, reduces the lycopene content of the red-fleshed genotypes. Thus the B^+/B and Del^+/Del genes probably

compete for a common precursor. B and Del, however, induces the production of large amounts of β - and α -ionone ring-containing carotenes only in fruits of the normal red genotypes; they have no effect on the carotenoid distribution in the rr fruits. Small amounts of α - and β -carotenes are produced when Del is introduced into the atat and tt genotypes (Tomes, 1969).

The recessive allele, gh, of the ghost gene causes an unstable chlorophyll deficiency in the plant. The stems, leaves, flowers and immature fruit may vary in the degree of pigmentation from completely white to yellow, or green. The ripe fruit, however, is yellow due to the yellow skin pigment. The fruit as well as the stems and leaves contain mainly phytoene. The total carotenoid content of the ripe fruit is approximately normal (Mackinney, Rick and Jenkins, 1956).

The crimson tomato is homozygous for the recessive allele, og^C (Thompson, Tomes, Wann, McCollum and Stoner, 1965). It increases the lycopene content of the ripe fruit concurrent with a loss of β -carotene.

The recessive sherry allele, sh, inhibits lycopene synthesis but not that of β -carotene (Zscheile and Lesley, 1967). Its effect is similar to the r allele in that it reduces the β -carotene content of the atat fruit.

All the genes which affect tomato fruit color are nuclear genes (Kirk and Tilney-Bassett, 1967), i.e. their inheritance is controlled by the nucleus in contrast to cytoplasmic or extranuclear genes whose inheritance is mediated by extranuclear genomes contained in such cell organelles as the plastids and mitochondria (Gibor and Granick, 1964).

C. Biosynthesis of Carotenoids

Carotenoids share the pathway common to all terpenoids from acetate to isopentenyl pyrophosphate and dimethylallyl pyrophosphate (Goodwin, 1965b,d). Mevalonic acid (Fig. 1) is the first metabolite that is specific to terpenoid biosynthesis in higher plants; once acetate is converted to mevalonic acid, it has no other fate in the system but to be utilized for terpenoid synthesis (Popjak and Cornforth, 1960). Certain microorganisms can utilize leucine as substrate for carotenoid biosynthesis (Chichester, Nakayama, Mackinney and Goodwin, 1955). Comprehensive reviews on terpenoid biosynthesis have been made by Goodwin (1965b, d) and more recently by Porter and Anderson (1967).

Phytoene is the first C_{40} compound formed from which all the other more unsaturated carotenoids are derived (Fig. 2). It is formed by the "tail-to-tail" dimerization of two molecules of geranylgeranyl pyrophosphate. The latter arises by successive "head-to-tail" condensation of isopentenyl pyrophosphate with dimethylallyl pyrophosphate, geranyl pyrophosphate, and farnesyl pyrophosphate (Fig. 1).

Dimethylallyl pyrophosphate is required for chain initiation (Agronoff, Eggerer, Henning and Lynen, 1959); isopentenyl pyrophosphate is required for chain elongation (Jungalwala and Porter, 1967). Varma and Chichester (1962) and Subbarayan, Kushwaha, Suzue and Porter (1970) reported the incorporation of isopentenyl pyrophosphate by tomato homogenate into lycopene, and by an enzyme preparation from spinach leaves into phyto-

Figure 1. Modified scheme for the biosynthesis of geranyl-geranyl pyrophosphate (Goodwin, 1965b,d).

(50)

Fig. 2. The biosynthesis of acyclic carotenoids (Goodwin, 1965b,d).

ene, phytofluene and lycopene. The conversion of geranylgeranyl pyrophosphate into phytoene has also been reported (Shah, Feldbruegge, Houser and Porter, 1968).

The condensation steps are stereospecific (Goodwin, 1969). The hydrogen atom eliminated in each step is originally the 4S hydrogen of mevalonic acid. The resulting 4,8,12 and 16 positions in geranylgeranyl pyrophosphate are all trans. In the dimerization of two molecules of geranylgeranyl pyrophosphate, one hydrogen atom is stereospecifically eliminated from each molecule. These are the 5S hydrogen of mevalonic acid. However, the double bond formed at the center of the phytoene molecule has the cis configuration (Jungalwala and Porter, 1965). Goodwin (1965) believes that the central double bond in the phytoene molecule is responsible for the absence of cyclic carotenoids with ring structure analogous to the steroid skeleton.

The exact mechanism of sequential introduction of double bonds has not been established. Rilling (1962) ruled out a hydroxylation mechanism for the introduction of double bonds on the basis that oxygen or an electron acceptor such as $K_3Fe(CN)_6$ is required for the conversion of phytoene and phytofluene to the less saturated members of the series.

Beeler and Porter (1962) reported the enzymic conversion of phytoene to phytofluene and to lycopene (Subbarayan et al., 1970). A cell-free enzyme preparation capable of converting all-trans geranylgeranyl pyrophosphate into phytoene, phytofluene, ζ -carotene, neurosporene and lycopene has been obtained from Phycomyces blakesleeana mutant by Lee and

Chichester (1969). Suzue (1961) reported the formation of δ -carotene from phytoene by a cell-free preparation of Staphylococcus aureus. The conversion of lycopene into cyclic carotenes containing α - and/or β -ionone rings by plastid preparations from higher plants has been reported (Decker and Uehleke, 1961; Wells, Schelble and Porter, 1964; Kushwaha, Subbarayan, Beeler and Porter, 1969; Hill and Rogers, 1969). Decker and Uehleke (1961) also observed the conversion of β -carotene into lycopene by leaf chloroplasts.

It has now been unambiguously demonstrated that the more unsaturated carotenoids are formed by dehydrogenation of phytoene. While it is already clear that two enzyme systems catalyze the formation of α - and β -ionone rings in carotenes and that the isomerizations of α -ionone to β -ionone, or vice versa, do not occur (Goodwin and Williams, 1965; Williams, Britton and Goodwin, 1967; Goodwin, 1969), the branch point at which ring closure starts is still unresolved and remains the most controversial point in carotenoid biosynthesis. Either neurosporene or lycopene could cyclize to form the α - and β -ionone residues of the carotene molecule. The possible pathways for these transformations are presented in Fig. 3.

In photosynthetic tissues, there appears to be a compartmentalization of terpenoid biosynthesis (Fig. 4). Terpenoids which are associated with the photosynthetic mechanism in the plastid are formed in greening tissue from CO₂ but not from exogenously supplied mevalonic acid (Mercer and Goodwin, 1962, 1963, 1964; Griffiths, Threlfall and Goodwin, 1964; Goodwin, 1965b,d). These include the carotenoids, the phytol side-chain of chlorophyll, the side-chain of plastoquinone, tocopherols and vitamin

Fig. 3. Alternative pathways for the biosynthesis of cyclic carotenes (Williams, Britton and Goodwin, 1967).

Scheme A

Fig. 3.--continued

Scheme B

Fig. 4. The proposed scheme for compartmentalization of terpenoid biosynthesis in higher plants (Goodwin, 1965b).

K_1 . The extrachloroplastidic terpenoids which include squalene, steroids, the side-chain of ubiquinone and other triterpenoids may be formed from mevalonic acid, presumably via isoenzymes of the terpenoid pathway. The plastid membrane is probably impermeable to mevalonic acid but not to CO_2 . A combination of enzyme segregation and specific membrane permeability was suggested by Goodwin (1965b,d) to account for the compartmentalization. Rogers, Shah and Goodwin (1967) described the possible existence of chloroplastidic and extrachloroplastidic mevalonic acid activating enzymes. They also showed (1966) the presence of two mevalonic acid kinases in both green and etiolated leaves differing only in their pH optima. The pH optimum for the chloroplast mevalonate kinase is at pH 7.5 while that of the extrachloroplastidic kinase is at pH 5.5.

Compartmentalization of terpenoid biosynthesis is a relatively common occurrence in biological systems. For example, natural rubber and other terpenoids are both synthesized from isopentenyl pyrophosphate; rubber is poly-cis while terpenoids, such as the carotenoids, are predominantly all-trans. The formation of isopentenyl pyrophosphate from mevalonic acid occurs in the serum of the latex whereas the polymerization of isopentenyl pyrophosphate takes place almost exclusively on the surface of existing rubber particles (Archer, Audley, Cockbain and McSweeney, 1963) catalyzed by an enzyme distributed between the hydrocarbon surface and the aqueous serum. The farnesyl pyrophosphate synthesized by the serum of rubber latex incorporated the 4R hydrogen of mevalonic acid, while the rubber formed contained only the 4S hydrogen (Archer, Barnard, Cockbain, Cornforth, Cornforth and Popjak,

1966). Thus in the rubber latex there are probably two enzymes responsible for the production of all-trans terpenoids and rubber; the trans terpenols are not intermediates in rubber biosynthesis.

II. MATERIALS AND METHODS

A. The Plant Material

Fruits of the "lutescent", high-beta and tangerine tomato lines were harvested from field-grown plants. The ghost plants were grown in the greenhouse. To obtain sufficient ghost fruits for the study, the plants were sprayed with a 3% solution of "Blossom-Set" (Science Products Co., Inc., Chicago, Illinois). The spray material contains as active ingredient 0.1389% β -naphthoxyacetic acid.

The "lutescent" tomato fruits were harvested and graded into the following stages of maturity: 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; and Stage 4. Full yellow, externally visible red coloration at the blossom-end and throughout the center of the fruit.

Each lot was soaked for 1 hour in a 10% solution of DMSO and ripened subsequently at 27-30⁰. The corresponding control lots were soaked in distilled water at the same time. Five fruits from each maturity group were removed each day and frozen at -17⁰. They were held in the freezer until analyzed.

The high-beta fruits in the DMSO experiment were harvested at the mature green stage. The fruits were similarly treated as above. The samples consisted of 3 fruits for each treatment. The samples were kept in the freezer (-17⁰) until analyzed.

In the CPTA experiments, the high-beta fruits were harvested at the turn stage. Ghost fruits containing small areas of green tissue were harvested as soon as the blossom-end turned yellow. Each fruit was injected with 1 ml. of either distilled water or a solution containing 4300 ppm of CPTA, and allowed to ripen at 25° for 12 days. Each treatment sample consisted of 12 high-beta fruits and 32 ghost fruits. Where tissue from the same fruit was used, each fruit was dissected. The red sections were combined and analyzed. The yellow sections of the same fruits served as the control.

Freshly harvested ripe tangerine tomato fruits were used in the isolation of poly-cis ζ -carotene.

B. The Chemicals

The solvents used in the extraction, chromatographic separation, and purification of the carotenoid pigments were all of analytical reagent grade. The solvents were redistilled prior to use. Glass-distilled pentane (b.p. 35-37°, Burdick and Jackson Laboratories, Inc., Muskegon, Michigan) was used in the purification of phytoene.

The adsorbent Sea Sorb 43 (MgO) and Hyflo Super Cel were obtained from Fisher Scientific Company, Medford, Massachusetts; the aluminum oxide, Woelm, used in the purification of the various fractions was obtained from Alupharm Chemicals, New Orleans, La. The silica gel F₂₅₄ was bought from Brinkmann Instruments, Inc., Westbury, N. Y. 11590, as precoated aluminum roll 0.2 mm thick.

The dimethyl sulfoxide was supplied by Crown Zellerbach Corp., Chemical Products Division, Camas, Washington. The CPTA was a gift of Amchem Products, Inc., Ambler, Pa. 19002.

Diethyl ether was freed from peroxides prior to use by percolating 200 ml. through 25 g. of neutral alumina, activity grade I.

C. General Procedure for Quantitative Analysis of Carotenoid Pigments of the "Lutescent", High-Beta, and Ghost Tomatoes.

1. Pigment Extraction

The samples were removed from the freezer. Each fruit was cut in half across the blossom-end to the stem-end; half of each fruit was returned to the freezer while the other half was cut into smaller pieces. The sample was homogenized in a Waring Blender. A 25-g. aliquot was weighed in a tared aluminum weighing dish and oven-dried at 70° until the weight of the material remained constant. A 50-g. aliquot of the homogenate was weighed for pigment extraction.

The pigment was extracted by blending the sample with acetone and petroleum ether (P.E.) under a continuous stream of N₂. The homogenate was filtered through a Buchner funnel attached to a vacuum pump. The extraction was repeated until the residue was colorless except for scattered flakes of yellow skin.

The filtrate was washed with distilled water to remove the acetone. The petroleum ether-soluble fraction was saponified with 200 ml. of 15% KOH in methanol. Saponification was accomplished by heating the mixture

for 15-20 min. on a steam table in the dark under a continuous stream of N_2 . The unsaponifiable fraction was transferred to petroleum ether by pouring the methanolic solution into a mixture of petroleum ether and a small amount of acetone in a separatory funnel. Addition of water transferred the pigment to the epiphase. The petroleum ether solution of the pigment was washed several times with large volumes of water until the hypophase was no longer basic. The solution was dried over anhydrous Na_2SO_4 and evaporated to dryness prior to chromatography. The ghost tomato fruit extract was chromatographed without prior saponification.

2. Chromatographic Separation

The "lutescent" and high-beta tomato extracts were taken up in a small amount of petroleum ether and chromatographed on a tightly-packed 18 x 160 mm. column of $MgO:Super\ Cel\ (1:2, w/w)$. The chromatogram was developed first with petroleum ether until the phytofluene band separated from the β -carotene band. When the two bands were separated, 1% diethyl ether in petroleum ether was added, followed by 2% diethyl ether in petroleum ether. The chromatogram was developed with this solvent system until the γ -carotene band separated from the lycopene band. At this point the concentration of diethyl ether was increased to 5%. The column was allowed to develop further before 2% acetone in petroleum ether was added. β -Zeacarotene separated from γ -carotene with this solvent system. Addition of 5% acetone further resolved the various bands.

The adsorbent plug was extruded after phytofluene was eluted and the various bands were cut from the plug. The pigments were eluted from

the adsorbent with a mixture of acetone and petroleum ether. Lycopene was washed from the adsorbent with ethanol in petroleum ether. The fractions were evaporated to dryness in a flash evaporator.

In the case of the ghost tomato extract, the chromatogram was initially developed with petroleum ether as before, followed by 100 ml. of 2% acetone in petroleum ether to elute the phytofluene band. The acetone concentration was increased to 5% which eluted the β -carotene band. The adsorbent plug was extruded and the other bands were cut from the plug. The pigments were eluted from the adsorbent as described above.

3. Purification Procedures

The crude phytoene fraction from the MgO:Super Cel column was purified by rechromatography on 15 g. of neutral alumina I in a 10 mm. glass column. Fifty ml. of pentane was percolated through the column to wash the adsorbent before the crude phytoene was added. The column was developed initially with 70 ml. pentane, followed by 150 ml. of 1% diethyl ether in pentane. Phytoene was eluted from the column with 2% diethyl ether in pentane. Five ml. volume fractions were collected with a fraction collector. Phytoene usually appeared between fractions 30 and 50.

Phytofluene was purified by rechromatography on a short column (ca. 18 x 60 mm.) of MgO:Super Cel (1:2, w/w). The phytofluene band was eluted from the column with petroleum ether.

ζ -Carotene was present but was not analyzed in experiments using the "lutescent" and high-beta tomatoes.

The β -zeacarotene obtained from the MgO:Super Cel column generally was not subjected to further purification because of the relatively small amount of the pigment. However, when it is contaminated with a substantial amount of a γ -carotene isomer as indicated by the appearance of a peak at 485 nm, the pigment was rechromatographed on basic alumina III. The pigment was eluted from the column with 1% diethyl ether in petroleum ether.

γ -Carotene was rechromatographed on neutral alumina III with petroleum ether as the developing solvent. In subsequent studies with CPTA, the γ -carotene fraction was purified routinely by thin-layer chromatography (TLC) on silica gel F₂₅₄ with 1% diethyl ether as the developing solvent. The less polar γ -carotene band was cut from the aluminum plate and eluted from the adsorbent with acetone-petroleum mixture.

The lycopene fraction was washed free of ethanol with distilled water. The petroleum ether solution was dried over anhydrous Na₂SO₄ and rechromatographed on a short column of neutral alumina III. The lycopene band was eluted from the column with petroleum ether.

The carotenoid concentration in the various fractions obtained from the MgO:Super Cel chromatogram of ghost tomato extract was estimated spectrophotometrically without further purification. The spectrum of the crude phytoene fraction did not show contamination. Phytofluene was contaminated by a compound absorbing at 278 nm which could not be removed by rechromatography on MgO:Super Cel. In any case, the contaminant did not affect absorption maxima of phytofluene at 367, 347 and 331 nm.

The ζ -carotene is the total of two ζ -carotenes differing in adsorptivity on the column. One band was adsorbed just above the β -carotene band, the other just below γ -carotene. The two fractions showed identical absorption spectra with $\lambda_{\max}$ at 423, 398 and 377 nm.

γ -Carotene was not analyzed routinely because of the small amount of the pigment. Its identity was verified when 500 g. of ghost tomato fruits were used. Lycopene was relatively free from contamination.

D. Extraction and Chromatographic Procedures for the Isolation of Poly-cis ζ -Carotene from the Tangerine Tomato

Thirty grams of tissue from freshly harvested tangerine tomato fruit were extracted with acetone-petroleum ether as usual under a continuous stream of N_2 . The extract was either chromatographed after washing the acetone out or saponified with 10% KOH in methanol. Saponification was accomplished by evaporating the petroleum ether in a flash evaporator to transfer the pigment into the methanolic solution. The unsaponifiable material was transferred to petroleum ether after 15 minutes, dried over anhydrous Na_2SO_4 , and chromatographed on a 22 x 360 mm. column of MgO: Super Cel (1:2, w/w). The chromatogram was first developed with 2% acetone in petroleum ether. The acetone concentration was increased to 5% and finally to 10%.

The adsorbent plug was extruded and the zone below the cis ζ -carotene band was cut together with the faint β -carotene zone. The pigments were eluted from the adsorbent with acetone-petroleum ether, evaporated to

dryness and rechromatographed on a 12 x 150 mm. column of neutral grade alumina II. The chromatogram was developed with increasing concentrations of diethyl ether in petroleum ether. The poly-cis ζ -carotene was eluted from the column together with the β -carotene band with 5% diethyl ether. The eluate was evaporated to dryness and purified by TLC on silica gel F₂₅₄ plates with petroleum ether as developing solvent. The yellow band adsorbed below the β -carotene band is the poly-cis ζ -carotene.

Alternatively, the eluate from the MgO:Super Cel column was first purified by TLC to remove the β -carotene. The zone containing the poly-cis ζ -carotene was eluted from the adsorbent with acetone-petroleum ether, evaporated to dryness, and rechromatographed on a short column of neutral grade alumina II to remove a contaminating β -zeacarotene-like pigment.

E. Pigment Identification

The various carotenoids were identified on the basis of their position on the MgO:Super Cel column and their absorption spectra (Davis, 1965).

F. Spectrophotometric Analysis

The absorption spectra were recorded from petroleum ether (b.p. 35-50°) solutions of the various carotenoids with a Cary 15 recording spectrophotometer, with the exception of phytoene whose spectra were recorded from either pentane or petroleum ether. The $E_{1\text{cm}}^{1\%}$ values and wavelengths used in the quantitative estimation of the various fractions are given below.

	$\lambda_{\max}$ nm	$E_{1\text{cm}}^{1\%}$
Phytoene	285	1250
Phytofluene	348	1350
ζ -Carotene	400	2270
Lycopene	472	3450
β -Zeacarotene	428	2570
γ -Carotene	462	3100
β -Carotene	451	2505

The amount of carotenoid in the solution was then calculated as follows:

$$\text{mg.} = \frac{\text{Absorbance} \times \text{Total Volume} \times \text{Dilution Factor}}{E_{1\text{cm}}^{1\%} \times 0.1}$$

Quantitative estimation of the poly-cis ζ -carotene was made from the equilibrium mixture of stereoisomers after the poly-cis pigment was subjected to iodine catalysis using the corresponding $E_{1\text{cm}}^{1\%}$ value for the all-trans ζ -carotene at 400 nm.

G. Iodine Catalysis

One to two drops of a dilute solution of iodine in petroleum ether was added to the pigment solution in a glass-stoppered quartz cell which was subsequently illuminated for ca. 10 sec. by exposure to direct sunlight, or by exposure to fluorescent light for ca. 30 sec. The absorption spectrum of the equilibrium mixture was then recorded.

III. DISCUSSION

The results with DMSO and CPTA on ripening detached tomatoes did not unequivocally prove that neurosporene is the only substrate for the cyclization of carotenes. It should be pointed out, however, that the data obtained are contrary to the suggestion that in the tomato fruit lycopene cyclizes to β -carotene (Lincoln and Porter, 1950; Porter and Lincoln, 1952; Tomes, Quackenbush and Kargl, 1958; Porter and Anderson, 1962).

A reappraisal of the results of studies on the genetics of the inheritance of several genes affecting tomato fruit color in conjunction with the results of ultrastructural studies on plastid development in the tomato fruit would lead to the conclusion that in the tomato fruit either separate or parallel pathways exist for the synthesis of β -carotene of the chloroplasts and the major carotenoids of the chromoplasts.

The apricot gene, for example, in the homozygous recessive form, atat, inhibits lycopene formation; the β -carotene level in the atat fruit is the same as that found in the at⁺at⁺ fruit (Jenkins and Mackinney, 1955; Tomes, Quackenbush and Kargl, 1958). The total carotenoid content of the apricot fruit is only about 10% of the total accumulation in the red genotype, suggesting that the pigment lost is mainly lycopene and the associated polyenes. A logical explanation of this observation is that β -carotene and lycopene are formed through separate pathways and that the at allele preferentially inhibits the lycopene pathway but not the other.

The B allele, analogously, has no effect on the β -carotene fraction in the tangerine tomato (Tomes, Quackenbush and Kargl, 1956). The β -carotene fraction which is not affected by the at and B alleles could be the structural carotenoid associated with the chlorophyll in the chloroplasts of the green tissue. This structural β -carotene may be that pigment required for the formation of the normal lamellar system of maturing chloroplast as well as for the protection of chlorophyll and the grana structure against photooxidative destruction (Wallis, 1967). It may also be the precursor for zeaxanthin which is involved in the protection of the photosynthetic apparatus against photodestruction (Blass, Anderson and Calvin, 1959; Yamamoto, Nakayama and Chichester, 1962). This β -carotene could be that fraction in the normal red (B⁺B⁺), intermediate-beta (BB), high-beta (BB), delta (De!De!), tangerine (tt), and high pigment (hphp) tomatoes which is not affected by ripening at 32° (Goodwin and Jamikorn, 1952; Tomes, Quackenbush and Kargl, 1956; Tomes, 1963); by DMSO treatment (Raymundo, Griffiths and Simpson, 1967, 1970) and irradiation (Burns and Desrosier, 1957).

Chloroplasts of green tissues of higher plants are morphologically similar (Kirk and Tilney-Bassett, 1967). They contain essentially the same pigment systems, namely, chlorophylls and carotenoids. The fine structure of the tomato fruit chloroplast is comparable to that of the leaf chloroplast (Lefort, 1957; Rosso, 1968). The chromoplasts of the ripe tomato fruit develop from the chloroplasts of the green fruit and do

not originate from undifferentiated plastids (Rosso, 1968; Harris and Spurr, 1969). The genes which determine tomato fruit color, with a few exceptions, do not affect the amount and/or kind of carotenoids in the leaves and green fruits. The genes that control carotenogenesis in the two organs appear to be inherited independently. Furthermore, mutations in the chloroplasts do not affect the pigment composition of the chromoplasts, and vice versa (Kirk and Tilney-Bassett, 1967). The leaves and green fruits of the tangerine tomato, tt, for example, do not contain the characteristic poly-cis carotenoids that accumulate in the ripe fruit. The low total pigment gene, r^+/r , does not affect the carotenoid content of the green leaves and green fruits even though in the low pigment tomato, rr, the ripe fruit contains only about 5% of the total carotenoids of the r^+r^+ fruit.

There is very little variation in carotenoid content of the green fruit among the various tomato fruit color mutants. In fact, the β -carotene content of the green fruit of the normal red (B^+B^+ , r^+r^+), the high-beta (BB, r^+r^+) and the low pigment (B^+B^+ , rr) mutants are essentially the same, i.e. 29 ± 6 , 25 ± 5 and 26 ± 6 $\mu\text{g/g}$ dry weight, respectively (Harris and Spurr, 1969). On ripening, more β -carotene is synthesized in the normal red (67 ± 7 $\mu\text{g/g}$) and in the high-beta mutant (675 ± 315 $\mu\text{g/g}$) while in the low pigment mutant (7 ± 1 $\mu\text{g/g}$) further synthesis of β -carotene is terminated and the structural β -carotene is degraded in the transition from chloroplasts to chromoplasts (Harris and Spurr, 1969a). This loss of

structural β -carotene may be characteristic of the r^+/r gene because substitution of rr for r^+r^+ in the normal red, tangerine, apricot and intermediate-beta lines resulted in the reduction of β -carotene concentration to the level found in the low pigment, rr , mutant (Jenkins and Mackinney, 1955; Mackinney, Rick and Jenkins, 1956; Tomes, Quackenbush and Kargl, 1958). Thus r^+/r gene probably controls the mechanism which derepresses the genes for the synthesis of proteins necessary for plastid transformation. The lutescent gene, l^+/l , might then impose a restriction on the extent to which β -carotene synthesis can progress. The stimulus is probably transmitted from the leaves because in detached Stage 2 fruits, β -carotene synthesis appeared to be still in progress at the termination of the experiment. In the more mature fruit (stage 3) the maximum amount of β -carotene was attained after 3 days of ripening (cf. Tables I. 1 and I. 2).

It is conceivable that abscisic acid, which inhibits the synthesis of gibberellins (Wareing, El-Antably, Good and Manuel, 1967) and isoprenoid plastid lipids such as carotenoids, chlorophylls and lipophilic benzoquinones (Mercer and Pughe, 1969; Lichtenthaler and Becker, 1970), influences this activation process in the plastids. Abscisic acid is present in the tomato fruit (Dorffling, 1970). Recently, Khudairi and Arboleda (1971) reported that lycopene synthesis increased in ripening detached tomato fruit treated with abscisic acid. Gibberellic acid, on the other hand, is known to inhibit lycopene synthesis in ripening tomato fruit (Dostal and Leopold, 1967).

An inhibitor with biological activity approaching that of abscisic acid has been isolated as one of the products of the photolysis of violaxanthin (Taylor and Burden, 1970). Taylor and Smith (1967) have suggested that carotenoids may be the natural precursors of abscisic acid.

Neither ripening at high temperature nor any of the common mutant genes which affect tomato fruit color could significantly reduce the residual β -carotene fraction. It is possible that the mechanism that controls its synthesis is associated closely with the control mechanism for chlorophyll synthesis (Bogorad, 1965; Lascelles, 1965). Any condition which results in an increase in chlorophyll synthesis will proportionately increase the synthesis of this β -carotene fraction. Thus the high pigment gene, hp^+/hp , increases the β -carotene concentration in both green leaves and fruit because the chlorophyll content increased (Baker and Tomes, 1964). Similarly, the β -carotene content of the ghost tomato, $ghgh$, varies with the amount of chlorophyll present in the tissue (Mackinney, Rick and Jenkins, 1956).

The parenchymatous cells of the locular tissue of the tomato fruit are ontogenetically different from the parenchymatous cells of the carpel walls (Cocking and Gregory, 1963). Differences in the carotenoid distribution between these two tissues have been observed (Jenkins and Mackinney, 1955; Thompson, Tomes, Wann, McCollum and Stoner, 1965). In the normal red tomato, there is more β -carotene but less lycopene in the locular tissue than in the pericarp. When $og^{c+}og^{c+}$ is replaced by og^cog^c as in

the crimson tomato, the concentration of lycopene in both tissues increased but the β -carotene level decreased so that the β -carotene content of the locular tissue dropped to about the same level as that found in the pericarp. The significance of these findings in relation to the biosynthesis of carotenoids in the tomato fruit is presently not known.

The synthesis of δ - and α -carotene fractions is enhanced by the introduction of the Del allele into the BB genotype (Tomes, 1967). Reciprocally, substitution of B for B⁺ in the DelDel genotype increases α -carotene synthesis but reduces δ -carotene accumulation. The reduction in δ -carotene accumulation on the incorporation of B into the DelDel genotype suggests that either δ -carotene is not the precursor for α -carotene contrary to the suggestion by Williams et al. (1967), or that the B allele shifts the equilibrium between δ - and α -carotene toward α -carotene. Such a shift would be predicted in the presence of B in the Del fruit if the function of the B enzyme is to mediate the formation of the β -ionone ring (Tomes et al., 1956; Tomes, 1964). The B enzyme is probably non-specific and would catalyze the formation of a β -ionone ring in the end of a carotene molecule with a completed chromophore, i.e. neurosporene, γ -carotene, δ -carotene, and for that matter, lycopene when made available; ζ -carotene, α -zeacarotene and β -zeacarotene would not be suitable substrates for the B enzyme. Alternatively, introduction of the Del allele into the BB genotype enhances α -carotene accumulation probably because more γ -carotene (Tomes, et al., 1954; Tomes, 1963; Raymundo, Griffiths and Simpson, 1970) is available as substrate into which Del can add an α -ionone ring.

A scheme for the biosynthesis of lycopene and the cyclic carotenes in ripening tomato fruit would be as shown in Figure 5. ϵ -Carotene accumulates to a limited extent in the DelDel genotype (Kargl et al., 1960); Tomes (1967, 1969) did not analyze for α -zeacarotene, β -zeacarotene and ϵ -carotene. The synthesis of α -zeacarotene is probably enhanced in the DelDel fruit, analogous to the effect of the B allele on β -zeacarotene accumulation in the BB fruit (Raymundo et al., 1970).

In summary, it would appear on the basis of the results of the present investigation in conjunction with the earlier genetic and ultrastructural studies on chromoplast development in the tomato fruit that the chloroplastidic β -carotene may be synthesized independently via parallel pathways with that of the chromoplast carotenoids. The β -carotene synthesized by ripening normal red tomato fruit is formed by a "carry-over" pathway from the green fruit. The large amounts of β -carotene which accumulate in ripening high-beta tomato is probably synthesized by the pathway operating during the transformation of chloroplasts to chromoplasts.

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Figure 5. A scheme for the biosynthesis of lycopene and the cyclic carotenes in the tomato fruit.

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