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# CONVERSION OF LYCOPENE TO β-CAROTENE BY PLASTIDS ISOLATED FROM HIGHER PLANTS

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**Abstract**—A significant incorporation of radioactivity into β-carotene was obtained when [$^{14}$C]- or [$^{3}$H]-lycopene was incubated with plastids isolated from French bean (*Phaseolus vulgaris*) seedlings or tomato fruit. The reverse reaction could not be demonstrated. The results support the view that in higher plants lycopene is a possible intermediate in the biosynthesis of β-carotene.

## INTRODUCTION

THE GENERALLY accepted scheme for the biosynthesis of carotenes proposed by Porter and Anderson[1] involves formation of the more unsaturated carotenoids by sequential dehydrogenation of phytoene (Fig. 1). The scheme accommodates evidence from many systems including higher plants[2] and mutants of microorganisms such as *Chlorella vulgaris*,[3] *Rhodopseudomonas spheroides*,[4] and *Neurospora crassa*.[5] The scheme is supported by direct evidence of interconversion of certain compounds; for example, the conversion of phytoene to phytofluene[6] and of isopentenyl pyrophosphate to lycopene[7] by tomato fruit plastids, and of phytofluene to β-carotene by chloroplasts from corn.[8]

However, one remaining point of contention is the identity of the immediate precursor of cyclic carotenes, both lycopene and neurosporene being possible substrates for the cyclization reaction. Decker and Uehleke[9] claimed that chloroplasts isolated from carrot leaves carry out the conversion of lycopene to β-carotene, an observation supported by Wells, Schelble and Porter[10] from studies in which γ-, δ- and β-carotenes were identified after administration of [$^{14}$C]lycopene to isolated tomato plastids or spinach chloroplasts. However, in normal red tomato fruit, high temperature inhibits the formation of lycopene but not of β-carotene,[11] suggesting that here β-carotene may not be synthesized from lycopene. There is evidence to support the suggestion that in tomato fruit two pathways exist for β-carotene synthesis; one, temperature-sensitive involving lycopene, and the other, temperature-insensitive, in which β-carotene is formed by the alternative pathway.[12,13]

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Recently and after the start of the present studies, the conversion of [15,15'-$^3$H]lycopene to neo-$\beta$-, $\alpha$-, $\beta$-, $\delta$- and $\gamma$-carotenes by spinach chloroplasts and by plastids from a high-beta tomato genetic line were reported.[14] Some of the results we report here, have been the subject of a preliminary communication.[15]

## RESULTS AND DISCUSSION

Tomato fruit were harvested at the half-ripened (orange) stage; this stage of ripening was defined experimentally by measurement of the chlorophyll, $\beta$-carotene and lycopene content of the fruit. This data has been recorded in a previous paper[16] for one of the varieties of tomato used (Eurocross); the kinetics of formation of pigments were similar for 'Moneymaker' tomatoes except that maximum levels of $\beta$-carotene and lycopene were somewhat less than half the corresponding values for 'Eurocross'. The difference may be a reflection of the different growth conditions for the two varieties; 'Eurocross' tomatoes were grown over the summer months in a greenhouse, while 'Moneymaker' tomatoes were grown in the autumn out-of-doors.

A preliminary comparison of incorporation of [2-$^{14}$C]MVA into terpenoids showed that 4 mm thick slices of tomato fruit incubated in petri dishes with solutions of radioactive metabolite were an appreciably better source of [$^{14}$C]lycopene than half-ripened tomato fruit injected with [2-$^{14}$C]MVA by syringe. The specific radioactivities of lycopene, $\beta$-carotene and sterol after administration of [2-$^{14}$C]MVA in a typical 24 hr incubation are given in Table 1.

TABLE 1. INCORPORATION OF MEVALONIC ACID-2-$^{14}$C INTO COMPOUNDS IN TOMATO FRUIT

| | Specific activities dis/min/$\mu$g* | | |
|---|---|---|---|
| Tissue used | Sterol | $\beta$-Carotene | Lycopene |
| 15 g Slices | 26·3 | 310 | 302 |
| 64 g Fruit | 9·5 | 102 | 13 |

* 5 $\mu$c of MVA administered.

Accordingly [$^{14}$C]lycopene or [$^3$H]lycopene was prepared as required from incubations of 25 $\mu$C DL-[2-$^{14}$C]MVA or 600 $\mu$C DL-[2-$^3$H]MVA with approx. 15 g tomato tissue slices for 24 hr at room temp. The [$^{14}$C]- or [$^3$H]lycopene so formed was isolated, recrystallized to constant specific radioactivity, and its radiochemical purity authenticated by TLC and radioautography.

For incubation of prepared radioactive lycopene or $\beta$-carotene with tomato tissue slices, two methods of solubilization of pigment were examined. Tween 80 was mainly used as solubilizing agent, and details are given in the methods section. However, we briefly report some work resulting from attempts to use dimethyl sulphoxide (DMSO) as a solubilizing agent. DMSO has been reported[17,18] to inhibit formation of many terpenoids in intact tomato fruit, including phytoene and lycopene, but apparently has little effect on $\gamma$-, $\beta$- or

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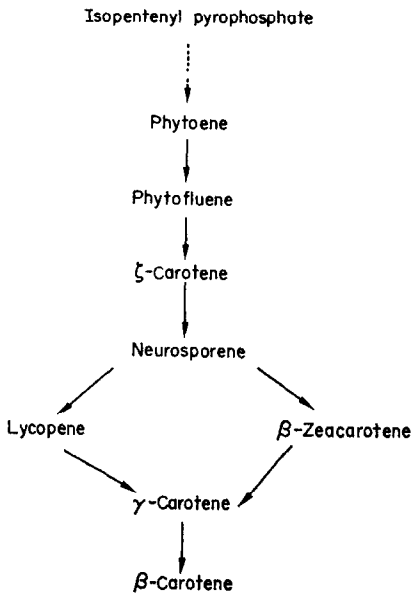


FIG. 1.

α-carotene synthesis. From such experiments, it has been concluded that separate compartmentalized pathways might exist for β-carotene synthesis via lycopene or β-zeacarotene respectively (see Fig. 1), and that DMSO affects only the latter pathway which is thought, in normal (low-beta) fruit, to be an inducible route occurring only in the ripening fruit. We believed therefore that for isolation of radioactive β-carotene, it would be advantageous to administer [2-$^{14}$C]- or [2-$^{3}$H]MVA to tomato fruit slices treated with DMSO, the subsequent isolation of radioactive β-carotene being facilitated by the presence of reduced lycopene levels in the fruit. However, in the presence of DMSO we found incorporation of [2-$^{14}$C]MVA into all terpenoid fractions studied to be much reduced, showing that synthesis (turnover) of pigments was severely diminished even though pigment levels remained more or less constant over the time of the experiment.

This data, presented in Table 2, demonstrated the inadvisability of using DMSO for subsequent experiments. The conditions employed in this study, viz. use of 4% DMSO and slices of tomato fruit, were markedly different to those used by Raymundo *et al.*[17,18] However, the study emphasises that under some conditions DMSO may act as a general inhibitor of terpenoid synthesis and that interpretation of data from experiments using it should be made with caution.

When [$^{14}$C]lycopene was incubated with plastid preparations from french bean seedlings or tomato fruit a significant incorporation of radioactivity into β-carotene was observed (Table 3). This incorporation reflects enzymatic conversion of lycopene to β-carotene since no conversion was affected by plastids previously subjected to treatment at 80° for 15 min. Similarly in controls where plastids were absent, but [$^{14}$C]lycopene present, no radioactivity was evident in zones of $R_f$ corresponding to β-carotene on subsequent TLC analysis.

Because of the long incubation periods used, (12–14 hr), we have borne in mind possible criticism that contaminating bacteria may have contributed to the observed activity.

TABLE 2. EFFECT OF DIMETHYL SULPHOXIDE (DMSO) ON INCORPORATION OF DL-[2-$^{14}$C]MEVALONIC ACID INTO TERPENOIDS OF TOMATO FRUIT

| Conditions | DMSO absent MVA added at 8 hr | DMSO present MVA added at 0 hr | DMSO present MVA added at 10 hr |
|---|---|---|---|
| Unsaponifiable lipid isolated (mg) | 43 | 44 | 45 |
| Unsaponifiable lipid (dis/min × $10^{-3}$) | 2304 | 1225 | 102 |
| Sterol isolated (mg) | 1·4 | 3·5 | 0·2* |
| Sterol (dis/min/mg) | 43,000 | 31,000 | 14,000 |
| β-Carotene isolated (μg) | 14 | 10 | 12 |
| β-Carotene (dis/min/mg) | 3900 | 1540 | 190 |
| Lycopene isolated (μg) | 120 | 70 | 122 |
| Lycopene (dis/min/mg) | 325,000 | 161,000 | 3900 |
| Phytoene (dis/min) | 3500 | 1400 | 100 |

* Low recovery after chromatographic separation.

All incubations (28°) were for 24 hr; the mixture contained 12 ml 0·1M Tris buffer, pH 7·5, in which was suspended approximately 15 g half ripe tomato slices. 6 μc DL-[2-$^{14}$C]MVA (4·82 mc/m-mole) was supplied at the times indicated. DMSO was used to a final concentration of 4% as shown.

TABLE 3. INCORPORATION OF [$^{14}$-C]LYCOPENE INTO β-CAROTENE BY PLASTIDS FROM FRENCH BEAN SEEDLINGS AND TOMATO FRUIT

| Source of plastids and incubation time (hr)* | Lycopene administered | | β-Carotene isolated | | | |
|---|---|---|---|---|---|---|
| | μg | dis/min × $10^{-2}$ | μg | dis/min | dis/min/mg × $10^{-2}$ | Conversion % |
| FB (10) | 390 | 139 | 12 | 410 | 340 | 2·9 |
| FB (20) | 390 | 139 | 15 | 390 | 260 | 2·8 |
| FB-HI (20) | 390 | 139 | 14 | < 10 | — | 0 |
| FB (15) | 205 | 60 | 44 | 550 | 125 | 9·6 |
| FB (24) | 410 | 120 | 85 | 1030 | 121 | 8·6 |
| FB-HI (15) | 205 | 60 | 46 | < 10 | — | 0 |
| T (6) | 390 | 139 | 25 | 390 | 155 | 2·8 |
| T (15) | 390 | 139 | 18 | 440 | 245 | 3·3 |
| T-HI (6) | 390 | 139 | 20 | < 10 | — | 0 |

* FB, French Bean; FB-HI, French bean plastids, heat inactivated; T, Tomato fruit; T-HI, Tomato fruit plastids, heat inactivated.

Each incubation mixture (25°) contained 5 mg Tween 80, 100 μmoles phosphate buffer pH 7·2, the given level of [$^{14}$C]lycopene and 1·0 ml of plastids (0·40–0·45 mg chlorophyll) in a final volume of 2·0 ml. The first three incubations were made under constant illumination (approx. 2200 lx), in remaining incubations the lower half of the incubation tube was enclosed in aluminium foil to exclude light. Plastids were subjected to ultrasonic treatment for 10 sec prior to incubation.

However, a number of factors indicate that the observed conversion is caused by the plastids themselves and not by any associated contaminating bacteria. Amongst these factors, the observation that in experiments with [$^{14}$C]lycopene no further activity was evident after prolonged incubation times compared to shorter incubations is but of secondary importance. A more satisfactory criterion comes from related studies (Hill and Rogers, unpublished observations) which have shown that in french bean seedlings grown under the laboratory

conditions used in the present work, the only significant contaminating bacteria present are *Pseudomonas fluorescens* and *Erwinia herbicola*. Though a few cells of these bacteria may be present in chloroplast preparations they could not contribute to the observed conversion of lycopene to β-carotene since analysis of cultures of the bacteria showed absence of carotenoids.

Study of the data in Table 3 indicates that conversion of lycopene to β-carotene by plastid preparations ceased for an undetermined reason some few hours after commencement of incubations, since in no case did prolonged incubation result in increased conversions of lycopene to β-carotene. Lack of some cofactor for the conversion might be responsible since no added cofactors were present in this series of incubations. Kushwaha *et al.*[14] have shown that NADP enhanced incorporation of lycopene to β-carotene by spinach chloroplasts and that there was an absolute requirement for FAD and a partial requirement for NADP when non-particulate preparations were used. This may be the reason why prolonged

TABLE 4. INTERCONVERSION OF [$^3$H]LYCOPENE AND β-CAROTENE BY PLASTIDS FROM FRENCH BEAN SEEDLINGS AND TOMATO FRUIT

| Source of plastids* | Lycopene administered | | β-Carotene isolated | | | |
|---|---|---|---|---|---|---|
| | μg | dis/min × $10^{-2}$ | μg | dis/min × $10^{-2}$ | dis/min /μg | Conversion % |
| FB | 60 | 4900 | 18·5 | 500 | 2700 | 10·2 |
| FB | 36 | 2600 | 25 | 250 | 980 | 9·5 |
| FB† | 43 | 3250 | 24 | 175 | 730 | 5·4 |
| FB‡ | 43 | 3250 | 25 | 178 | 710 | 5·5 |
| FB§ | 43 | 3250 | 20 | 403 | 2000 | 12·9 |
| FB-SI | 43 | 3250 | 31 | 5·2 | 17 | 0·2 |
| FB-HI | 43 | 3250 | 30 | 0·2 | — | 0 |
| T | 36 | 2600 | 25 | 182 | 730 | 7 0 |
| T-HI | 36 | 2600 | 29 | 64 | 220 | 2·4‖ |
| | β-Carotene administered | | Lycopene isolated | | | |
| | μg | dis/min | μg | dis/min | dis/min /μg | |
| FB | 7·5 | 30,300 | 33 | 150 | 4·5 | 0·5 |
| FB-HI | 7·5 | 30,300 | 36 | 170 | 4·7 | 0·5 |

* FB, french bean; T, tomato fruit; SI, inactivated by sonication (60 sec); HI, inactivated by heating.

† Incubation at 45° instead of 25°.

‡ In Tris buffer, pH 9·0.

§ In Acetate buffer, pH 5·5.

‖ Appreciable residual activity still present due to possible misiming of heat treatment.

Incubations were 12 hr (T) or 14 hr (FB) in duration. Except where indicated all incubations were made at constant illumination (2200 lx) under $N_2$ at 25°; each incubation mixture contained 5 mg Tween 80, 100 μmoles of phosphate buffer, pH 7·5, 30 μmoles NADP, 0·01 μmole $MgCl_2$, the given level of [$^3$H]lycopene and 1·0 ml of plastids (0·40–0·45 mg chlorophyll) in a final volume of 2·0 ml. Plastids were subjected to ultrasonic treatment for 10 sec prior to incubation.

ultrasonication (Table 4) leads to loss of ability of plastids to carry out the conversion of lycopene to β-carotene.

Because of the low specific radioactivity (36,000 dis/min/mg) of the lycopene isolated from tomato fruit supplied with [2-$^{14}$C]MVA the total counts in the recrystallized β-carotene isolated from incubation mixtures was usually only of the order of 400 dis/min. Although reliable assessment of this quantity of radioactivity could be made it was recognized that lycopene of higher specific radioactivity would be advantageous. Consequently in a later series of experiments [2-$^{3}$H]MVA was used and this gave [$^{3}$H]lycopene of specific radioactivity of the order of $7 \times 10^6$ dis/min/mg which was used in further incubations with plastid preparations. The results obtained are summarized in Table 4.

Significant conversions of lycopene to β-carotene could be demonstrated. Change in pH of the incubation mixture showed that conversion was enhanced at low pH's. Thus under directly comparable conditions, conversion at pH 5·5 was over twice that at pH 9·0; in this connection it is interesting to note that one mechanism proposed[14] for conversion of lycopene to β-carotene involves initial protonation. It has been suggested that the lycopene pathway for synthesis of β-carotene is temperature sensitive.[12,13] However, in our experiments a reduced but still significant conversion occurred at 45° indicating that, *in vitro* at least, part of the pathway involving conversion of lycopene to β-carotene is not readily affected by increased temperatures.

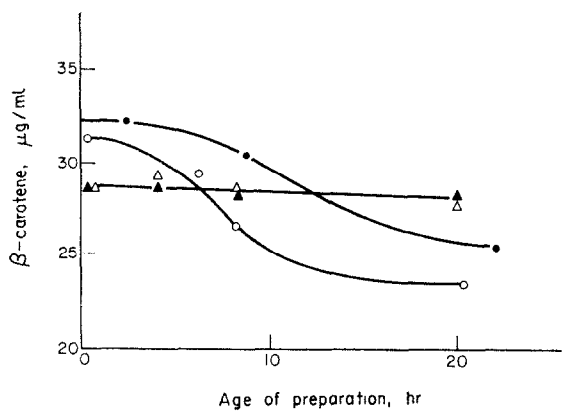


FIG. 2. LEVEL OF β-CAROTENE IN PLASTID PREPARATIONS MAINTAINED IN THE PRESENCE OR ABSENCE OF LYCOPENE.

Plastid preparations (A and B) from french bean seedlings were exposed to the conditions used in experimental incubations. 1·0 ml aliquots were taken at intervals for analysis of β-carotene.

A —○—○—○—Normal plastid suspension
—●—●—●—Plastids with 200 μg/ml lycopene added

B —△—△—△—Plastid suspension – heat treated
—▲—▲—▲—Plastids – heat treated, with 200 μg/ml lycopene added.

We were unable to demonstrate a net synthesis of β-carotene in the incubations because of the loss of β-carotene by action of degradative enzymes or, possibly, by non-enzymic decomposition. However, it was possible to show increased levels of β-carotene in french bean plastid preparations incubated with lycopene, compared to controls where lycopene was absent (Fig. 2). After heat inactivation of plastids this difference was undetectable, the level of extractable β-carotene remaining approximately constant over the suc-

ceeding 24 hr, lending credence to the view that the loss of β-carotene observed in normal incubations might be due to enzymic degradation.

During the isolation of β-carotene by TLC after incubations with radioactive lycopene, the presence of other possible intermediates or products of the conversion was sought. However, because of the small quantities present and consequent difficulty in purifying the pigments, no identification could be made. Identification of neo-β, α-, β- and γ-carotenes were reported in similar studies by Kushwaha *et al.*[14] but the yields were not given nor any data by which the amount of plastids used in their incubations could be determined, so direct comparison with the results given here was not possible.

When [$^3$H]β-carotene was incubated with french bean plastids some 0·5% of the added radioactivity was subsequently identified in lycopene isolated from the incubation mixture and recrystallized. However an almost identical conversion was observed in a control experiment with a heat inactivated plastid preparation indicating that either the β-carotene prepared may only have been some 99·5% radiochemically pure, or that some non-enzymatic conversion of β-carotene to lycopene had occurred. In our view, therefore, this data does not justify the conclusion that β-carotene has been converted to lycopene by such incubation.

The data presented does show that plastids from higher plants are able to convert lycopene to β-carotene. No intermediates or other pigments were found in quantities which would permit unequivocal identification or reliable assay. Therefore, the possibility does exist that lycopene was converted to β-carotene not directly via γ-carotene, but through neurosporene, β-zeacarotene and γ-carotene (Fig. 1). Against this suggestion, it can be argued that the initial step would involve hydrogenation, while the typical reactions leading to formation of the unsaturated carotenes are all dehydrogenations. Moreover, Kushwaha *et al.*[14] using high-beta tomatoes were unable to demonstrate neurosporene as a product under conditions where appreciable quantities of other pigments related to β-carotene were formed from lycopene.

While the present studies demonstrate the capacity of higher plant plastids to form β-carotene from lycopene it cannot yet be concluded that this is the typical or even the major route by which β-carotene is formed *in vivo*. Other evidence,[19,20] has been presented to support neurosporene as the cyclization substrate; the present work merely confirms that the candidature of lycopene for this role cannot be dismissed. Alternative routes for cyclization may exist in different organisms or even, in cases such as tomato, within the same tissues. The control mechanisms which dictate which route is operative at any given time are still a matter for speculation.[18]

## EXPERIMENTAL

*Solvents.* Light petroleum (b.p. 40–60°), benzene (A.R.) and $Et_2O$ (A.R.) were dried (Na) and redistilled; the $Et_2O$ was distilled over reduced Fe immediately before use.

*Chromatographic absorbents.* Aluminium oxide, Woelm, anionotropic grade was obtained from M. Woelm, Eschwege, Germany; Kieselgel G from E. Merck A.-G., Darmstadt, Germany.

*Radioisotopes.* DL-[2-$^{14}$C]mevalonic acid lactone (4·82 mc/mmole) and DL-[2-$^3$H]mevalonic acid lactone (93 mc/mmole) were obtained from The Radiochemical Centre, Amersham, Bucks.

*Plant material.* Greenhouse grown 'Eurocross' tomatoes or 'Moneymaker' tomatoes grown out-of-doors or bought locally were used. The mature green fruit was harvested at 50–60 days after anthesis.

*Phaseolus vulgaris* (french bean) plants were grown from seed (Carters' Lightning variety, Carters Tested Seeds, London, S.W.20).

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*Administration of radioisotopes to intact tomato fruits.* 1 ml of an aqueous solution of the radioisotope was injected by hypodermic into the parenchyma tissue of the tomato through the stem scar. During subsequent incubation (24 hr), the wound produced by the needle was kept moist by a drop of $H_2O$ to prevent desiccation. Tomatoes were illuminated (laboratory conditions—approx. 2000 lx) at room temp for the experimental period.

*Isolation of plastids.* Tomato parenchyma tissue was homogenized in an approx. equal volume of 0·1 M Tris buffer, pH 8·2 containing 0 001 M EDTA and 0·001 M mercaptoethanol, for 60 sec in a Waring blender, solutions at 2–4°. The homogenate was filtered through several layers of cheesecloth and centrifuged at 4000 *g* for 30 min. The plastid pellet was resuspended, usually in 0·1 M phosphate buffer, pH 7·2, before incubation with radioactive substrate.

Intact chloroplasts from french bean leaves were prepared by discontinuous sucrose density gradient centrifugation according to Leech [21] The chloroplast pellet was resuspended in 0·1 M phosphate buffer, pH 7·2, before use.

*Solubilization of pigments for incubation with plastids.* Lycopene or β-carotene was dissolved in $Et_2O$ or benzene and added to 1 0 ml of buffer containing 5 mg Tween 80. Excess organic solvent was removed under a stream of $N_2$ before addition of the plastid preparation.

*Extraction and separation of terpenoids.* General methods employed for extraction of lipid, saponification and extraction of unsaponifiable lipid, and separation of terpenoids have been described [16]

When levels of pigments in incubations with plastid preparations were very low, the total lipid extract was subjected to TLC on Silica gel. EtOAc (7 5%) in light petroleum was used for development, the β-carotene zone ($R_f$ 0·75) running just ahead of lycopene. The eluted β-carotene zone was contaminated with lycopene and was subjected to repeated chromatography on Kieselgel G plates using benzene:light petroleum (1:9 v/v) for development, until chromatographically pure. This was recrystallized to constant specific radioactivity in the usual way. Lycopene was always present in sufficient quantity for purification by normal procedures.[16]

*Recrystallization of β-carotene and lycopene.* The methods described previously were followed with the addition that recrystallized products were subjected to TLC followed by radioautography of the developed chromatogram to check the radiochemical purity of the sample.

*Estimation of chlorophyll.* Chlorophyll was determined as chlorophyll (a + b) by the method of Arnon.[22]

*Radioassay.* Samples were assayed in a Nuclear Enterprises Scintillation Counter. The procedures and precautions taken during radioassay of samples have been previously described.[16]

*Acknowledgements*—We gratefully acknowledge financial support from the Science Research Council for purchase of the Scintillation Counter used in this and related investigations.