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INCORPORATION OF [2-^{14}C]GLYOXYLATE, [2-^{14}C]ACETATE AND [2-^{14}C]MEVALONIC ACID INTO TERPENOIDS DURING RIPENING OF TOMATO FRUIT

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Abstract—The incorporation of radioactivity from [2-^{14}C]glyoxylate, [2-^{14}C]acetate and [2-^{14}C]mevalonic acid into terpenoids during ripening of detached tomato fruit has been studied. Incorporation of [2-^{14}C]-glyoxylate into β-carotene and lycopene, on a specific radioactivity basis, decreased as the fruit ripened. In contrast, incorporation from both [2-^{14}C]acetate and [2-^{14}C]mevalonic acid into β-carotene increased throughout the ripening process and into lycopene increased during early stages of ripening. Incorporation of all three metabolites into sterols decreased rapidly during the early ripening of the fruit but appeared to be relatively constant during later stages of ripening. The results are discussed in relation to possible changes in metabolism and accessibility of sites of pigment synthesis as the chloroplasts change during senescence to chromoplasts.

INTRODUCTION

PRESENT evidence[1,2] has led to the view that the control of terpenoid biosynthesis in developing seedlings is achieved mainly by a combination of enzyme segregation within the cell and comparative impermeability of the intracellular membranes to terpenoids and their immediate precursors. Experimental evidence has led to the view that acetyl-CoA, an obligatory intermediate in chloroplast terpenoid biosynthesis, is formed within the chloroplast from carbon dioxide. A route proposed for this biosynthesis[3] involves glyoxylate as one of the early intermediates formed from CO_2 and it has been shown that radioactivity from [2-^{14}C]glyoxylate is effectively incorporated into β-carotene by illuminated etiolated maize seedlings

Acetyl-CoA is converted into isopentenyl pyrophosphate, the common biological isoprene unit, by a well established pathway involving mevalonic acid (MVA). In this metabolic sequence mevalonic acid is the first intermediate specific to terpenoid biosynthesis.[4] The first intermediate specific to carotenoid biosynthesis is phytoene, a C_{40} unit formed by tail-to-tail condensation of geranylgeranyl pyrophosphate. Phytoene is converted to lycopene, the major carotenoid (87 per cent) in the normal red tomato,[5] by sequential desaturation. Some doubt still exists as to whether the smaller amounts (7 per cent) of β-carotene in the normal red tomato are formed from lycopene or whether these two major carotenoids in tomato fruit are formed by divergent routes from a common precursor.[6]

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It has become evident from many studies that the difficulty of incorporation of intermediates in the known biosynthetic pathway into chloroplast terpenoids in excised but otherwise intact plants can often be explained by difficulty of penetration of the metabolite to the site of synthesis within the chloroplast. For example, Goodwin[7] showed that [2-^{14}C]acetate and [2-^{14}C]MVA were poorly incorporated into β-carotene in maize seedlings under conditions where extensive incorporation occurred into sterols (extrachloroplastidic). The lack of incorporation of radioactivity from [2-^{14}C]acetate is puzzling since acetate is known to penetrate the chloroplast membrane and to be used inside the chloroplast for fatty acid formation.[8] In red tomatoes both acetate[9] and mevalonic acid[10] are good precursors of lycopene. These differences between maize seedlings and tomato fruit may be due to changes in permeability of the chloroplast bounding membrane as the chloroplasts senesce during ontogeny to form chromoplasts, in which chlorophylls and most terpenoid quinones have largely disappeared but carotenoids are very actively synthesized. Senescence of the plastid might also be accompanied by quantitative changes in metabolic potentiality. To test these proposals we have investigated the incorporation of three precursors of chloroplast terpenoids into β-carotene and lycopene at different stages during ripening of detached tomato fruits.

RESULTS AND DISCUSSION

We investigated the incorporation of [2-^{14}C]glyoxylate, [2-^{14}C]acetate and [2-^{14}C]-mevalonate into unripened (green), half-ripened (orange) and ripened (red) detached tomato fruit. The stages were defined experimentally by measurement of chlorophyll, β-carotene and lycopene content of the fruit during the ripening process (Fig. 1).

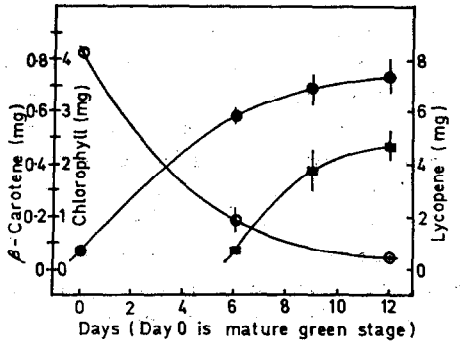

FIG. 1. VARIATION IN PIGMENT CONTENT DURING RIPENING OF TOMATO FRUIT.

The mature green stage of the fruit was reached 50–60 days after anthesis. The weights recorded are mg pigment/100 g fresh wt. tomato fruit. Each point was derived from four to six experiments. The vertical bars are ±S.E.M. in length.

—o—o—o— Chlorophyll
—●—●—●— β-Carotene
—■—■—■— Lycopene

Though some variation was observed in the levels of the pigments the overall trends of formation or disappearance of individual pigments were clearly distinguishable.

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When the incorporation of radioactivity from the labelled metabolites into terpenoids was examined the results shown in Table 1 were obtained. For convenience results are given both in terms of total radioactivity (dpm) and specific radioactivity (dpm/mg). The absolute values of specific activity will be influenced by both pool sizes and turnover rates, unknown variables for the system studied. However, knowledge of total radioactivity incorporated and of the relative specific activities of intra- and extra-plastidic terpenoids at different stages of ripening enables assessment of the effectiveness of different labelled substrates as precursors of β-carotene and lycopene during the ripening process.

It will be seen from the Table that the incorporation of radioactivity from [2-^{14}C]glyoxylate into unsaponifiable lipid is somewhat greater in half-ripened fruit than in green fruit.

TABLE 1. INCORPORATION OF [2-^{14}C]GLYOXYLATE, [2-^{14}C]ACETATE AND [2-^{14}C]MEVALONIC ACID INTO STEROLS, β-CAROTENE AND LYCOPENE OF RIPENING DETACHED TOMATOES

Substrate†	[2-^{14}C]glyoxylate			[2-^{14}C]acetate			[2-^{14}C]MVA		
Stage of fruit*	UR	HR	R	UR	HR	R	UR	HR	R
Weight of fruit (g)	108	110	88	108	74	126	94	68	104
Unsaponifiable lipid isolated (mg)	50	63	57	55	56	76	41	52	63
Unsaponifiable lipid (dpm × 10^3)	170	245	43	980	1,620	840	6,274	4,051	3,416
Sterol isolated (mg) (4-desmethyl and 4,4'-dimethyl sterols)	2·3	2·8	3·0	2·0	1·9	4·1	2·6	1·9	3·2
Sterol (dpm/mg)	4,360	250	390	32,600	12,250	16,000	791,000	34,000	20,000
β-Carotene isolated (μg)	65	650	905	100	470	800	70	360	705
β-Carotene (dpm)	1,470	9,000	9,450	4,200	22,900	60,200	1,050	21,600	37,100
β-Carotene (dpm/mg)	22,600	13,850	10,450	42,000	48,700	75,250	15,000	60,000	52,600
Lycopene isolated (μg)	—	690	3,200	—	530	5,120	—	310	4,850
Lycopene (dpm)	—	7,700	2,600	—	21,300	23,000	—	21,000	36,400
Lycopene (dpm/mg)	—	11,200	800	—	40,200	4,500	—	67,700	7,500
Lycopene plus β-carotene (dpm)	1,470	16,700	12,050	4,200	44,200	83,200	1,050	42,600	73,500

The substrates, in aqueous solution, were injected by aseptor disposable syringe into the detached fruit through the stem scar.

Fruit were then illuminated (approx. 200 ft cdl.) at 23–27° for 24 hr.

* UR, Unripe (green) tomatoes; HR, Half-ripe (orange) tomatoes; R, Ripe (red) tomatoes.

† 15 μc [2-^{14}C]sodium glyoxylate (16 mc/m-mole); 30 μc]2-^{14}C]sodium acetate (38 mc/m-mole); 30 μc [2-^{14}C]MVA (4·82 mc/m-mole).

However, when the fruit has ripened there is substantially less incorporation despite the large incremental synthesis of lycopene during the later stages of ripening. Very little of the radioactivity incorporated into the unsaponifiable lipid is associated with sterol especially when the fruit is ripening. In contrast to the sterol fraction there is, on a specific radioactivity basis, significant incorporation of radioactivity from [2-^{14}C]glyoxylate into β-carotene in green fruit; this incorporation decreases as the fruit ripens. Similarly, the specific radioactivity of lycopene is much higher in half-ripened fruit than during the terminal stages of ripening. Because of the large incremental synthesis of β-carotene and lycopene during ripening this change is obscured when figures for total radioactivity incorporated are considered though even here the decreased incorporation in ripened compared to half-ripened fruit is evident. In a second series of experiments with another tomato variety (Moneymaker) similar results were

obtained except that the specific radioactivity of the β-carotene and lycopene were higher (51,000 dpm/mg β-carotene in green fruit after administration of 10 μc [2-^{14}C]glyoxylate)

The results with [2-^{14}C]glyoxylate are in marked contrast to those obtained when [2-^{14}C]-acetate or [2-^{14}C]MVA were administered. With [2-^{14}C]acetate the maximal incorporation of radioactivity into unsaponifiable lipid occurs in half-ripened fruit. The incorporation into sterol is far greater than that observed when [2-^{14}C]glyoxylate was administered even taking into account the increased radioactivity supplied. When chloroplastidic terpenoids were assayed for radioactivity it was found that on both a total radioactivity and specific radioactivity basis increased incorporation of acetate occurred as the fruit ripened. The increase is particularly evident when total radioactivity data are considered.

In general [2-^{14}C]MVA behaved similarly to [2-^{14}C]acetate though there were some significant differences. Thus the very high incorporation of radioactivity into unsaponifiable lipid observed with green fruit steadily decreased as the fruit ripened. Incorporation into sterol was exceptionally high in green fruit but had substantially decreased even in partially ripened fruit; thereafter little change in incorporation was evident. This observation was confirmed in a second series of experiments. Incorporation of radioactivity into β-carotene increased as the fruit began to ripen but on a specific radioactivity basis then remained relatively constant in half-ripened and ripened fruit. Incorporation into lycopene differed in that on a specific radioactivity basis substantially less incorporation occurred in ripened fruit than in half-ripened fruit. The complicating factor in this case may be the high quantity of lycopene present in ripened fruit. These data were similar to those obtained when [2-^{14}C]acetate was administered. Comparison of the data for the three metabolites shows incorporation into sterol decreases as the fruit ripens due presumably to a decreased turnover of sterol. The low incorporation of glyoxylate into sterol is expected since the pathway implicating glyoxylate as a precursor of terpenoids appears to be confined within the chloroplast.[3] In green fruit MVA appears to be a considerably better precursor of sterol than does acetate; this is in marked contrast to the situation in greening etiolated maize where both substrates are incorporated to approximately the same extent.[3]

When incorporations into chloroplast terpenoids are considered it is seen that incorporation of radioactivity from [2-^{14}C]glyoxylate on a specific radioactivity basis decreases significantly as the chloroplast senesces. The data for [2-^{14}C]MVA, to which the higher plant chloroplast is relatively impermeable, indicates that during ripening (senescence) the sites of biosynthesis of chloroplast terpenoids become more accessible to administered metabolites. However, [2-^{14}C]glyoxylate, which in contrast to MVA readily enters chloroplasts, is not incorporated to greater extent as ripening proceeds for though the total radioactivity incorporated does increase initially during the ripening process the specific radioactivity of β-carotene and lycopene show a progressive decrease. From this it would appear that during ripening the increased permeability of the chloroplast membranes to glyoxylate is offset by a corresponding reduction in metabolic potentiality for forming acetyl-CoA and hence β-carotene and lycopene, from glyoxylate. The preservation during senescence of the route from acetate to chloroplast terpenoids is indicated by the increased incorporation of acetate, and mevalonate, into β-carotene even in fully ripened fruit.

Interpretation of the results with [2-^{14}C]acetate are complicated by the knowledge that though the higher plant chloroplast is relatively permeable to acetate[8] it is not normally utilized in chloroplast terpenoid biosynthesis when administered to illuminated etiolated maize seedlings.[7] It has been suggested that acetate entering the chloroplast is preferentially used for fatty acid biosynthesis and is not available for chloroplast terpenoid formation. In the case

of unripe tomato fruit, however, on a specific radioactivity basis effective incorporation of radioactivity from [2-^{14}C]acetate into β-carotene does occur and the incorporation does not increase appreciably during the first half of the ripening process. This data might suggest that the fatty acid synthesizing ability of chloroplasts in green tomatoes might be far less than in maize chloroplasts. However, it must be borne in mind that in green tomato fruit we are dealing with mature chloroplasts while in studies with illuminated etiolated maize seedlings chloroplasts in the process of formation are being studied. We do have some data from other experiments to suggest that tomato fruit chloroplasts prior to the mature green stage show more similarity to developing maize chloroplasts. Clarification of this point will await data, to our knowledge not available at present, on the fatty acid synthesizing ability of tomato plastids.

It should be emphasized that the data for the three metabolites given in Table 1 does take into account the relative efficiency of the individual metabolites as precursors of β-carotene or lycopene. For example, all forty carbons of β-carotene should be labelled from [2-^{14}C]-glyoxylate whilst only twenty carbon atoms are labelled from [2-^{14}C]acetate. [2-^{14}C]-Mevalonate contributes only eight carbon atoms per molecule of β-carotene and in this case it must also be borne in mind that only one isomer of the DL-mevalonate supplied is utilized in formation of β-carotene.

We interpret the data summarized in Table 1 therefore, as indicating that during ripening of tomato fruit (*a*) sterol turnover decreases; (*b*) the chloroplast, which in the unripe fruit is relatively permeable to glyoxylate and acetate, becomes permeable also to MVA; (*c*) metabolic activity of the glyoxylate route of chloroplast terpenoid biosynthesis decreases whereas (*d*) the biosynthetic route from acetate (acetyl CoA) via MVA to chloroplast terpenoids remains operative.

EXPERIMENTAL

Solvents. Light petroleum (b.p. 40–60°), benzene (A.R.) and diethyl ether (A.R.) were dried over sodium wire and redistilled; the diethyl ether was distilled over reduced iron immediately before use.

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

Radioisotopes. [2-^{14}C]Sodium acetate (38 mc/mM), [2-^{14}C]sodium glyoxylate (16 mc/mM) and DL-[2-^{14}C]-mevalonic acid lactone (4·82 mc/mM) were obtained from The Radiochemical Centre, Amersham, Bucks.

Fruits. Greenhouse grown "Eurocross" tomatoes were used in this study. The mature green fruit were harvested at 50 to 60 days after anthesis.

Administration of radioisotopes. Radioisotopes were administered by injecting 1 ml of an aqueous solution of the radioisotope into the parenchyma tissue of the tomato through the stem scar. During subsequent incubation (24 hr) the wound produced by the hyperdermic needle was kept moist by a single drop of distilled water to prevent desiccation. Tomatoes were illuminated (laboratory conditions—approx. 2200 lx) at room temp. (23–27°) during the ripening process and for the 24 hr subsequent to injection of radioisotope.

Extraction and Separation of Terpenoids

Extraction of lipid. Tomato fruit were homogenized with four volumes of acetone in a Waring Blendor; the acetone was filtered off and the residue was re-extracted with acetone in the case of unripe fruit and with acetone: *n*-hexane (v/v) for ripened and partially ripened fruit. After the initial tissue disruption by Waring Blendor, homogenisation was carried out using the ultraturrax homogenizer (Hudes Mechanising Co. Ltd., London, W.1). This procedure was repeated until the residue was white except for fragments of red skin in the case of half-ripened and ripened fruit.

The bulked acetone or acetone–hexane extracts were exhaustively extracted with ether (4 × 125 ml portions). The combined ether extracts were freed from acetone with water and then dried over anhydrous Na_2SO_4 (20 g/100 ml extract) for 30 min. The Na_2SO_4 was filtered off and washed with ether; the ethereal extract was evaporated to small volume at 60° under N_2. During all extractions the vessel containing the lipid was enclosed in tin-foil to minimise photo-decomposition of pigments.

Saponification and extraction of unsaponifiable lipid. The method of Goodwin[11] was used except that saponification was carried out in the cold room (4°) overnight and not with heating.

Separation of terpenoids. The unsaponifiable components were first separated into crude fractions by column chromatography on alumina (Brockmann grade 3) supports (10 g absorbent/100 mg unsaponifiable components). Elution by stepwise fractionation with light petroleum containing ether yielded two fractions. The fraction eluted by 100 ml 1% ether in petroleum ether per 10 g absorbent, contained mainly squalene, colourless polyenes and carotenoids; that eluted by 14% ether in petroleum ether contained mainly sterols, phytol and pentacyclic triterpenes. When lycopene was present in appreciable quantities the volume of 1% ether in petroleum ether was increased two-fold. The crude carotenoid and sterol fractions were concentrated and purified further by TLC.

The carotenoid fraction was evaporated to dryness and the pigments dissolved in a small volume of cyclohexane before application to a TLC plate (Kieselgel G). Benzene: light petroleum (1:9 v/v) was used for development of the chromatogram. The β-carotene (R_f 0·4) and lycopene (R_f 0·2) zones, visible as coloured bands, were eluted and rechromatographed separately. The β-carotene and lycopene zones were then eluted, evaporated to dryness and the pigments dissolved in a known volume of petroleum ether (β-carotene) or benzene (lycopene). The amount of pigment present was calculated from $E^{1\%}_{1cm}$ 2505 (450 mμ) and $E^{1\%}_{1cm}$ 3182 (486·5 mμ) respectively. The identity and purity of the two pigments was also confirmed from their absorption spectra in petrol–ether, hexane and $CHCl_3$. Finally the β-carotene and lycopene were crystallized to constant specific radioactivity in the presence of authentic carrier β-carotene (routinely) or lycopene (when necessary).

The sterol fraction containing 4 desmethyl sterols and 4,4'-dimethyl sterols was obtained from the 14% ether in petroleum ether fraction eluted from the alumina column by methods described elsewhere.[3]

Recrystallization of β-carotene and lycopene. β-Carotene or lycopene was dissolved in a minimum volume of benzene at room temp. Excess of methanol was added and the solution placed in the cold room (−25°) overnight. The crystals were collected by filtration (under slight vacuum using a micro sintered glass funnel) and washed twice with 5 ml aliquots of cold (0°) methanol. The crystals were finally dissolved in a suitable solvent such as petroleum ether (5–25 ml according to activity) for ^{14}C assay, and a small volume (0·1–0·2 ml) was diluted appropriately for spectrophotometric assay. The latter step was omitted if unlabelled carrier β-carotene or lycopene had been added previously. After the sample had been counted the solvent was removed under N_2 at 30° and the remaining pigment recrystallized; this process was repeated until a constant specific activity was attained.

Typical figures for recrystallizations are given here for convenience; in general it was found that specific radioactivities of the pigments was often significantly lowered by the first recrystallization but thereafter usually remained constant after the second and subsequent recrystallization.

Recrystallization of β-carotene after administration of [2-^{14}C]glyoxylate to unripe tomatoes (Table 1, column 1).

β-Carotene isolated 65 μg

Specific radioactivity (from TLC)	83,850 dpm/mg
Crystallization	38,000 dpm/mg
1st Recrystallization	22,500 dpm/mg
2nd Recrystallization	21,500 dpm/mg

Recrystallization of lycopene after administration of [2-^{14}C]MVA to ripe tomatoes (Table 1, column 9).

Lycopene isolated 4850 μg

Specific radioactivity (from TLC)	19,400 dpm/mg
Crystallization	9600 dpm/mg
1st Recrystallization	7800 dpm/mg
2nd Recrystallization	7500 dpm/mg

Estimation of chlorophyll. Chlorophyll was determined as chlorophyll (a + b) by the method of Arnon.[12]

Radioassay. Samples were assayed in a Beckman LS-200B scintillation counter. Samples were dissolved in 5 ml of toluene and when necessary decolourized under u.v. light (Phillips 300W ultraviolet lamp) before addition of 5 ml of scintillator containing 10 g 2,5-diphenyloxazole/l. toluene. Samples were decolorized for 20–120 min (depending on the amount of pigment present) with the u.v. light approximately 6 in. above and at

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a 45° angle to the glass counting vial. Progress of decolorization was determined by visual inspection. The decolorized samples were stored overnight at 0° in the dark before ^{14}C assay. If decolorized samples are assayed immediately on removal from the u.v. light spurious counts are recorded on ^{14}C-assay. Absence of spurious counts can be confirmed from a series of ^{14}C-assays at intervals of several hours.

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