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CAROTENOID BIOSYNTHESIS IN SOME MAIZE MUTANTS

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Abstract—A number of maize mutants with blocks in carotenoid synthesis have been examined. They all contained phytoene; others contained in addition phytofluene and one phytofluene, ζ-carotene and neurosporene. Lycopersene could not be detected in any mutant. The polyenes are located in the 600 *g* fraction. [2-^{14}C]Mevalonate is much more effectively incorporated into phytoene and phytofluene in the mutants than it is into β-carotene in normal maize. In this respect the "chloroplasts" in the mutant strains resemble chromoplasts in fruit and roots (carrots) rather than normal chloroplasts, the membranes of which are relatively impermeable to mevalonic acid.

INTRODUCTION

WHEN etiolated seedlings are illuminated they rapidly turn green owing to the development of the colourless plastids into functional chloroplasts containing, amongst other newly synthesized compounds, chlorophylls and carotenoids. If such seedlings are exposed to [2-^{14}C]mevalonate (a specific isoprenoid precursor) as they turn green, little activity appears in β-carotene or the terpenoid side chains of chlorophyll, plastoquinone, tocopheryl quinone or vitamin K_1, although these compounds are being rapidly synthesized; on the other hand, sterols, pentacyclic triterpenoids and ubiquinone are strongly labelled. An exactly opposite situation is observed if $^{14}CO_2$ is the labelled substrate; the chloroplast terpenoids are strongly labelled whilst the sterols show only slight activity. These observations have led to the view that terpenoid synthesis in developing chloroplasts is regulated by a combination of enzyme segregation and specific membrane permeability.[1] When chloroplasts change into chromoplasts as in ripening fruit, such as tomatoes, the situation appears to change and exogenous [2-^{14}C]mevalonic acid is effectively incorporated into the carotenoids which are being actively synthesized.[2–5] Similarly, plastids in carrot root slices incorporate [2-^{14}C]mevalonate into β-carotene in the absence of marked synthesis.[3, 6, 7] There is clearly a difference between mevalonate metabolism by plastids, whether derived from chloroplasts or not, and fully developed chloroplasts. It was important to see if plants in which chloroplasts had been altered by mutations responded differently to mevalonic acid. Through the kindness of Dr. D. Robertson (University of Iowa) we have been able to examine a small number of such mutants derived from commercial strains of maize.

[1] T. W. GOODWIN, in *Biosynthetic Mechanism in Higher Plants* (Edited by J. D. PRIDHAM). Academic Press, London (1965).
[2] T. W. GOODWIN, *Advanc. Enzymol.* **21**, 295 (1959).
[3] G. D. BRAITHWAITE and T. W. GOODWIN, *Biochem. J.* **76**, 1, 5, 194 (1960).
[4] E. A. SHNEOUR and I. ZABIN, *J. Biol. Chem.* **234**, 770 (1958).
[5] A. E. PURCELL, G. A. THOMPSON and J. BONNER, *J. Biol. Chem.* **234**, 101 (1959).
[6] V. V. MODI and D. K. PATWA, *Enzymologia* **23**, 27 (1961).
[7] J. W. PORTER and D. ANDERSON, *Arch. Biochem. Biophys.* **97**, 520 (1962).

RESULTS

Nature of Polyenes and Pigments

The nature and amount of polyenes present in mutant W_3 grown in darkness or in dim light for 6 days are recorded in Table 1. The components phytoene, phytofluene, ζ-carotene and neurosporene were identified by co-chromatography with authentic specimens and by

TABLE 1. THE NATURE OF THE CAROTENOID POLYENES OF MAIZE MUTANT W_3 GROWN IN DARKNESS OR IN LIGHT

		Seedlings grown in darkness (30 seedlings, fresh wt. 1·6 g, unsaponified 11·5 mg)		Seedlings grown in 0·5 lm (15 seedlings, fresh wt. 1·8 g, unsaponified 13·1 mg)	
Component identified*	R_f	Absorption maxima (light petrol) (nm)	Amount (μg)	Absorption maxima (light petrol) (nm)	Amount (μg)
Phytoene	0·41	275, 285, 296	3·5	275, 282, 297	3·0
Phytofluene	0·31	330, 347, 367	2·1	331, 348, 367	0·5
ζ-Carotene	0·06	385, 400, 425	0·7	387, 400, 425	trace
Neurosporene	Origin	413, 445	trace	445	faint trace

5–6-day seedlings: unsaponifiable matter first chromatographed on alumina (Grade III) and light petrol fraction rechromatographed on thin-layer plates (Kieselgel G) with light petrol as developer.

* Squalene (R_f, 0·69) was always detected.

u.v. and visible spectrophotometry and exist in seedlings germinated under both cultural conditions; phytoene had previously been reported by Anderson and Robertson.[8] It is clear that not only is there an almost complete block of carotenoid synthesis in this mutant (about 3 μg/g tissue compared with 25 μg/g in normal etiolated maize and 40 μg/g in green maize tissue, but that there is also a qualitative difference in the polyenes produced. In etiolated maize fully dehydrogenated xanthophylls such as lutein and violaxanthin preponderate; these are biosynthetic end-products. In mutant W_3 however, it is the compounds representing the first steps in carotenoid biosynthesis, phytoene → neurosporene, which are present. These are not detectable in etiolated seedlings and occur only in minute amounts in green tissues (phytoene and phytofluene 0·28 and 0·15 μg/g fresh weight in *Vicia sativa*;[9] ζ-carotene and neurosporene have not yet been detected). Lycopersene, the C-40 analogue of squalene (C-30) which has been suggested as the first C-40 compound formed in carotenoid biosynthesis,[10] could not be detected. Synthetic lycopersene runs with an R_f of 0·55 in our thin-layer system, and as we can detect 0·05 μg lycopersene on a plate it is possible to say that if it is present in the mutants it can only exist at a concentration less than 0·15% of that of phytoene. This same lower limit is indicated from isotope experiments (see next section).

Polyenes found in other white-seeded mutants grown in darkness or dim light were: albino W_3 60-1201-7⊕n; phytoene and phytofluene, similar concentration as in W_3—no ζ-carotene or neurosporene; W_3 59-3024-25⊕n phytoene and phytofluene; and W_3-ch/2 60-1047-19⊕ phytoene. The concentration of phytoene was about the same or slightly greater than in W_3. In no case was lycopersene detected. Yellow seeds were also produced by

[8] I. C. ANDERSON and D. S. ROBERTSON *Plant Physiol.* **35**, 531 (1960).
[9] E. I. MERCER, B. H. DAVIES and T. W. GOODWIN, *Biochem. J.* **87**, 317 (1963).
[10] E. C. GROB, K. KIRSCHNER and F. LYNEN, *Chimia* **15**, 308 (1961).

many of these mutants and these, when germinated in light of moderate intensity (500 lm) or grown in the dark and then exposed to light, produce green seedlings. However, under these conditions they still produced the same polyenes as the etiolated seedlings and as their white-seeded counterparts. Mutants which gave yellow seeds were W_3-ch/2 60-1047-19⊕ (phytoene and phytofluene) and W_3 59-3024-25⊕(phytoene). A yellow-seeded mutant W_3 60-1046-3⊕n also produced phytoene and, in traces phytofluene.

Localization of the Polyenes

Yellow seeds of mutant W_3-ch/2 60-1047-19⊕ were grown in darkness for 6 days and then transferred to light (500 lm) for 24 hr. The seedlings were homogenized in sucrose buffer and separated by differential centrifugation at 2° into three fractions which sedimented at 600 *g*; 20,000 *g* and 105,000 *g* respectively. The major proportion of the phytoene was associated with the green "chloroplast" pellet, 600 *g*, only traces were found in the other fractions. On the other hand only traces of squalene were detected in the chloroplast fraction but considerable amounts were located in the 105,000 *g* fraction together with the sterols. The same experiments were carried out with seedlings of yellow seeds of mutant W_3 59-3024-25⊕n and mutant W_3 60-1046-3⊕n which had been grown in the dark and greened up and the same distribution was observed. In one experiment with yellow mutant W_3 59-3024-25⊕n, a homogenous chloroplast fraction was obtained by gradient centrifugation; again the major terpene hydrocarbon was phytoene, accompanied by small traces of squalene. It can be concluded, therefore, that the small amounts of carotenoid polyenes which these mutants synthesize are specifically located in the "chloroplast" fraction.

Incorporation of [2-^{14}C]*Mevalonic Acid into the Polyenes*

Six-day seedlings of albino mutant W_3-ch/2 60-1047-19⊕ grown either in darkness or dim light (0·5 lm) were excised and placed for 24 hr in water containing [2-^{14}C]mevalonic acid. The polyene fraction was separated and purified and the radioactivity of phytoene compared with that of squalene. Table 2 shows that incorporation into phytoene was marked and was nearly 50% of the incorporation into squalene. The total sterols were also examined and found to have the same specific activity. No significant counts were obtained in the zone of the chromatogram where lycopersene would have occurred. As our methods would easily record a count of 20 disintegrations/min above background, this result means that if lycopersene were present, its concentration must be less than 0·15% of that of phytoene. This confirms the observations on direct analysis (see previous section).

In further experiments, the incorporation of [2-^{14}C]mevalonate into phytoene and squalene was compared in seedlings of mutant W_3-ch/2 60-1047-19⊕ grown in dim light (0·5 lm) and in darkness and gave a similar picture. The results are not recorded here.

In experiments in which [2-^{14}C]mevalonic acid was incorporated into seedlings from yellow mutants during exposure to light which were then homogenized and subjected to differential centrifugation, it was found, as in the case of the non-isotope experiments, that the counts in phytoene were concentrated in the "chloroplast" fraction whilst those in squalene segregated with the 105,000 *g* fraction.

It is important to compare these results with those with normal maize under comparable conditions. In greening etiolated seedlings the amount of [2-^{14}C] incorporated is somewhat greater (9·2%) (Table 3) compared with those in Table 2, but the incorporation into β-carotene is insignificant and very much less than into squalene and the sterols; in the darkness squalene and sterol were still strongly labelled.

TABLE 2. COMPARISON OF INCORPORATION OF [2-^{14}C]MEVALONATE IN ALBINO W_3-ch/2 60-1047 GROWN IN DARK AND DIM-LIGHT CONDITIONS

Fraction	Plants grown in dark (6·62 g)			Plants grown in light (6·04 g)		
	Weight (mg)	Disintegrations/min	Disintegrations/min/mg	Weight (mg)	Disintegrations/min	Disintegrations/min/mg
Lipid	19·8	166,800	8420	20·1	178,200	8050
Unsaponified	10·7	166,680	15,580	12·7	171,000	13,470
Sterol-free unsaponified	9·7	74,490	7680	8·3	79,500	9580
Sterols	2·10	80,100	38,140	1·97	91,500	46,450
Light petrol fraction	1·9	17,100	9000	1·7	12,150	7150
50% E/P	5·2	56,160	10,800	3·6	48,480	13,470
100% Ether	2·1	1320	630	1·7	1680	990
Waxy material	—	—	—	—	—	—
Squalene	—	8210	—	—	6930	—
"Lycopersene zone"	—	0	0	0	0	0
Phytoene	0·13	5820	45,000	0·095	3400	36,000
Origin		3000	—	—	1800	—

20 seedlings germinated for 6 days, excised and exposed to DL-mevalonic acid ($9·2 \times 10^5$ disintegrations/min) for 24 hr.

TABLE 3. THE INCORPORATION OF [2-^{14}C]MEVALONIC ACID INTO THE UNSAPONIFIABLE MATERIAL IN NATIVE MAIZE

Fraction	Total incorporation (disintegrations/min $\times 10^{-3}$)	Specific activity (disintegrations/min $\times 10^{-3}$)
β-Carotene	24	47·7
Free sterols	2100	96
Sterol esters	500	294

6-day seedlings, excised and greened for 24 hr in presence of DL-mevalonic acid, 6 μc.

DISCUSSION

The "chloroplasts" of the yellow-seeds maize mutants examined in this investigation contain small amounts of chlorophyll and are sedimented at 600 *g*. They do not, however, contain the normal complement of carotenoids, β-carotene, lutein, violaxanthin and neoxanthin, but only traces of the partly saturated carotenoid precursors which normally do not occur in such relatively high concentrations if at all (Table 1). Apart from this difference there must also be a marked difference in membrane structure of the "chloroplasts" of the mutants compared with normal chloroplasts. In normal chloroplasts the incorporation of [2-^{14}C]mevalonic acid into β-carotene, and other terpenoid chloroplasts is very much reduced compared with the incorporation into squalene and sterols (Table 3). This is because mevalonate cannot easily penetrate the intact chloroplast; Rogers *et al.*[11] showed that it was not converted into mevalonate phosphate and pyrophosphate by intact chloroplasts prepared in non-aqueous solvents, but that it was actively converted into these compounds by preparations of non-aqueous chloroplasts which had been disintegrated by sonication. In

[11] L. J. ROGERS, S. S. P. SHAH and T. W. GOODWIN, *Biochem. J.* **96**, 7P. (1965).

the mutant strains the situation is clearly different, because although the phytoene and phytofluene are located in the plastids, exogenous [2-^{14}C]mevalonic acid is incorporated effectively into these polyenes, the extent of incorporation being of the same order as into the extraplastidic squalene. Compare Tables 2 and 3 which show that in contrast to the mutant strains normal maize incorporates [2-^{14}C]mevalonic acid only weakly into β-carotene but very effectively into squalene. Thus from the point of view of terpenoids the plastids of the mutants appear to be similar to chromoplasts in fruit (e.g. tomatoes), which are senescent chloroplasts, and to the chromoplasts in carrot root.

It is probably significant that once again a series of mutants has been obtained in which carotenoid synthesis is blocked and which accumulate phytoene not lycopersene (see Goodwin,[12] for other instances). This is an additional piece of evidence to support the growing view that phytoene and not lycopersene is the first C-40 compound formed in carotenoid biosynthesis.

EXPERIMENTAL

Growth of Plants

Two groups of mutant maize were used; white seed W_3 mutants which on germination produced albino seedlings, albinism being associated with the white endosperm of the seed, and yellow seed W_3 mutant which produced green seedlings. They were dusted with a commercial preparation (I.C.I. Seed Saver) and planted in trays containing moist vermiculite or soil. All seeds were germinated for 5–6 days before use, the white mutants either in darkness or in dim light (0·5 lm), and the yellow mutants in darkness or in light (500 lm).

As mevalonate cannot penetrate the root system of maize,[3] the seedlings were excised at the first internode and placed in an aqueous solution of [2-^{14}C]mevalonate (5–10 μc) for 24 hr either in darkness or light (0·5 or 500 lm).

Disruption of Tissue and Purification of Intracellular Organelles

The seedlings were cut into small pieces with scissors and homogenized in ice-cold buffer containing sucrose (0·5 M), KH_2PO_4 (0·1 M), EDTA (0·01 M) at pH 7·2 in a blender operated at full speed for 30 sec,[13] all operations being carried out in a cold-room at 0–4°. The resulting homogenate was filtered through cheese-cloth (6–8 layers), which removed the large cell debris, and the filtrate centrifuged in a Spinco Model L, refrigerated centrifuge. Three fractions were obtained: (*a*) 600 *g*, which contained the bulk of the intact chloroplast particles, (*b*) 20,000 *g*, which contained fragmented chloroplast and mitochondria, and (*c*) 105,000 *g*, the so-called microsomal fraction. The supernatant which remained after the final spin was also investigated.

The 20,000 *g* and 105,000 *g* fractions were partly purified by suspending the pellets in buffer and by resedimentation at the appropriate mean centrifugal force for the appropriate times. The 600 *g* fraction was purified by density gradient centrifugation.[14] The gradient consisted of a mixture of two solutions of glycerol–sucrose buffer, 25:75 (v/v) and 60:40 (v/v) respectively. Equal volumes (10 ml) of each were mixed in a centrifuge tube, the mixture having a mean density of 1·102, and the gradient allowed to stand to equilibrate for an hour at 0–4° before use. The density of the lower solution was 1·154 and that of the upper layer 1·060.

[12] T. W. Goodwin, in *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. Goodwin). Academic Press, London (1965).
[13] O. Holm-Hansen, N. G. Pon, K. Hishida, V. Moses and M. Calvin, *Physiol. Plant* **12**, 475 (1959).
[14] W. O. James and V. S. R. Das, *New Phytol.* **56**, 325 (1957).

The crude 600 *g* fraction was suspended in 5 ml of buffer and carefully pipetted on to the gradient, ensuring that the layers were not disturbed in the process, and submitted to a mean centrifugal force of 600 *g* for 12 min. The "purified" intact chloroplasts formed a dark green band at the interpenetration layer of the gradient, the yellow to pale-green upper layer containing the contaminating smaller particles and the lower sediment the aggregates and particles larger than the chloroplasts, such as starch grains. The chloroplast layer was carefully pipetted out, resuspended in buffer and sedimented at 600 *g* for 12–15 min.

Extraction of Terpenoids

The seedlings, or the fractions obtained by differential centrifugation, were extracted with acetone in a blender and the resulting suspension filtered by suction through a sintered glass funnel. The residue was extracted again with acetone and the process repeated four or five times. The bulked acetone extracts were diluted with water and extracted several times with ether. The ethereal extracts were washed several times with water, dried over anhydrous Na_2SO_4 for 30 min and evaporated to dryness under N_2. The resulting lipid was examined spectrophotometrically for chlorophyll, and then saponified by the usual procedure and the unsaponifiable material (unsap.) extracted into ether.[15] After washing the ethereal extract containing the unsap. free from alkali, the solution was dried over Na_2SO_4 and taken to dryness under N_2.

Removal and Estimation of Sterols

The sterols were removed from the unsap. by precipitation as their digitonides[16] by dissolving the unsap. in aqueous 95% (v/v) ethanol and heating to boiling, to which was added a boiling solution of 2% (w/v) digitonin in aqueous 90% (v/v) ethanol, and boiling continued until a white precipitate appeared. Precipitation of the sterol digitonides was completed overnight at 0°, the digitonides sedimented by centrifugation and purified by washing with mixtures of ethanol and ether. The washings were added to the supernatant.

The sterols isolated as their digitonides were dissolved in glacial acetic acid and a known amount treated with the Liebermann–Burchard reagent; the colour was developed at 25° for 33 min in the dark and the extinction measured at 620 nm in a Unicam SP 500 spectrophotometer. The ratio of the intensity of green colour produced with the reagent, to the quantity of sterol digitonide is the same as for the free sterol.[7] The weight of sterol was calculated from a standard curve prepared from β-sitosterol.

Chromatography of Sterol-free Unsaponifiable Matter

The supernatant and washings from the removal of the sterol digitonides was treated with ether which caused precipitation of the excess digitonin, which was removed by centrifugation. The supernatant solution was dried over Na_2SO_4 and taken to dryness under N_2, then dissolved in light petrol (free from aromatic hydrocarbons for purposes of u.v. spectroscopy) and chromatographed on columns of alumina (acid washed) weakened with water to Brockmann Grade III. The columns were developed with light petrol and increasing proportions of ether in light petrol (E/P), using 100 ml of each solvent per 10 g column. The light petrol fraction was occasionally re-chromatographed on alumina (Brockmann Grade V), and the phytofluene band detected with an u.v. lamp.

[15] T. W. GOODWIN, In *Modern Methods of Plant Analysis* (Edited by K. PAECH and M. V. TRACEY) Vol. 3, p. 372 (1955).
[16] R. SCHOENHEIMER and H. DAM, *Z. Physiol. Chem. Hoppe-Seyler's* **215**, 59 (1933).
[17] W. E. DAVIES, Ph.D. Thesis, University of Wales (1963).

Suitable samples of the light petrol fractions obtained by chromatography on alumina were examined by thin-layer chromatography on Kieselgel G in a modified saturation-chamber[18] with light petrol as developing solvent. The chromatograms were exposed to iodine vapour and the resulting brown zones noted. Larger samples (occasionally diluted with non-radioactive authentic lycopersene) were applied to the chromatoplate as a long strip rather than as a single spot, in order to prevent overloading. Authentic compounds were separated simultaneously on the chromatogram, and the narrow vertical strip of adsorbent containing the reference compounds, removed from the plate by means of transparent adhesive tape (Sellotape) and exposed to iodine vapour to reveal the position of the hydrocarbons. By returning the strip to its original place on the chromatoplate, the position of the unstained zones could be marked. The squalene, lycopersene, phytoene and all other staining zones were then carefully scraped off the plate, transferred to small centrifuge tubes and extracted with 2×3 ml $CHCl_3$ and once with 3 ml of ethanol. The bulked extracts from each zone were evaporated to dryness under N_2 and measured for radioactivity; in the case of phytoene and phytofluene and other polyenes when present the extracts were also examined spectroscopically. The hydrocarbons were frequently re-run on chromatoplates, eluted and radioassayed by the same means.

Measurement of Radioactivity

Radioassays were carried out with a Nuclear Measurements Corp. (Chicago, U.S.A.) PCC-10A proportional counter, coupled to a Panax (100C EHT) fast scaler unit, at a geometrical efficiency of 35 per cent. All measurements were carried out by applying 0·1–0·2 ml of a suitable solution of the fraction to a stainless steel disk, previously prepared with lens tissue, and the solvent evaporated by irradiation with i.r. lamps. The lens tissue ensured an even spread of the fraction to be assayed.[19] The usual corrections for background and self absorption were made.

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[18] B. H. Davies, *J. Chromatog.* **10**, 518 (1963).
[19] J. Glover, W. M. F. Leat and R. A. Morton, *Biochem. J.* **66**, 214 (1957).