Incorporation of [^{14}C]Carbon Dioxide and [$2\text{-}^{14}\text{C}$]Mevalonic Acid into Terpenoids of Higher Plants during Chloroplast Development

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1. The incorporation of $^{14}\text{CO}_2$ and DL-[$2\text{-}^{14}\text{C}$]mevalonic acid into various terpenoids in developing chloroplasts in a number of seedlings has been studied. 2. β -Carotene and phytol (from chlorophyll) tend to be heavily labelled from $^{14}\text{CO}_2$, whereas sterols and β -amyirin are only slightly labelled; with DL-[$2\text{-}^{14}\text{C}$]mevalonic acid the situation is reversed. 3. The incorporation of $^{14}\text{CO}_2$ into terpenoids is dependent on the stage of chloroplast development, whereas that of mevalonic acid is independent of chloroplast development. 4. The uptake of $^{14}\text{CO}_2$ into β -carotene and phytol in mature chloroplasts is very low in monocotyledons but somewhat greater in dicotyledons. 5. The results are discussed in relation to the view that terpenoid biosynthesis in developing chloroplasts is regulated by a combination of enzyme segregation and specific membrane permeability.

Etiolated seedlings contain relatively large amounts of sterols but only traces of carotenoids, mainly xanthophylls, and no chlorophylls, although protochlorophyll is sometimes present in small amounts. On illumination, etiolated seedlings rapidly become green; this is caused by the development of proplastids, already present in the dark-grown seedlings, into functional chloroplasts with a simultaneous accumulation in the chloroplasts of chlorophylls, carotenoids, plastoquinones, tocopherol and tocopherylquinones, and vitamin K_1 (see Goodwin, 1965). All these compounds either are wholly terpenoid in nature (carotenoids) or contain terpenoids as part of their molecule (phytol in chlorophylls, tocopherols and vitamin K_1 ; solanesol in plastoquinone A). Thus the formation of terpenoids is a vital aspect of the development of functional chloroplasts. The ability of etiolated seedlings to synthesize sterols means that the first common steps in the biosynthetic pathway of all terpenoids (e.g. from acetate to isopentenyl pyrophosphate) are already functional in the dark; the problem is how light channels these precursors away from sterol synthesis into the synthesis of the compounds listed above, which may be termed 'plastidic terpenoids'. The present paper reports one of a series of investigations into this problem which have resulted in the formulation of a hypothesis to explain the regulation of terpenoid synthesis in greening seedlings (Goodwin, 1965).

Goodwin (1958) observed that, if etiolated excised maize seedlings were placed in a solution of [$2\text{-}^{14}\text{C}$]mevalonic acid and illuminated, then the

label was rapidly taken up into sterols but not into β -carotene, although there was a rapid net synthesis of this compound during the experiment. Conversely, under the same conditions $^{14}\text{CO}_2$ was specifically incorporated into β -carotene but only slightly into sterols. This phenomenon has now been examined in much greater detail in a number of monocotyledons and dicotyledons. Monocotyledons and dicotyledons were both examined because there are marked differences in the way their chloroplasts develop. For example, the proplastids of monocotyledons, unlike those of dicotyledons, enlarge in darkness (Granick, 1961), and the formation of a continuous lamellar system precedes grana formation in monocotyledons, but in dicotyledons grana regions appear first (Wettstein, 1959).

Part of this work has already been briefly reported (Treharne, Goodwin & Mercer, 1964).

EXPERIMENTAL

Plants. Maize (South African White Horse-Tooth, hybrid no. 59C/421) was obtained from Gunson's Ltd., London; oats (Manod S235) were from the Welsh Plant Breeding Station, Aberystwyth; peas (var. Onward) and dwarf French beans (var. Lightning) were from Carter's Seeds Ltd., London. The seeds were dusted with a commercial preparation of fungicide (Seed Saver; Imperial Chemical Industries Ltd.) and germinated at 28° in the light or in specially darkened cupboards at 28° in trays of vermiculite or light loamy soil (John Innes no. 1 mixture).

Lettuce (Webbs Wonderful) plants were obtained locally and as they were too mature to transplant they were maintained hydroponically in a modified Hoagland A-Z medium

Fig. 1. Apparatus for illuminating excised seedlings in an atmosphere of $^{14}\text{CO}_2$.

(P. Randall, personal communication). The plants were supported on a waxed board with their roots dipping into the nutrient solution, contained in earthenware vessels, which was continuously aerated over the culture period. The waxed board was fitted tightly to the vessel to minimize bacterial or fungal infection of the nutrient solution.

Administration of radioactive compounds. [$2\text{-}^{14}\text{C}$]Mevalonate was introduced into seedlings by placing the stems, excised at the internode connecting root and stem, into an aqueous solution of labelled substrate; mevalonic acid is not significantly absorbed by the roots (Goodwin, 1958).

Seedlings and leaf slices were exposed to $^{14}\text{CO}_2$ in an apparatus that maintained constant temperature and intensity of illumination (Fig. 1). The leaf preparation was placed in a round-bottomed flask (100 ml. capacity) with a long neck (F). The flask was connected to the gas line and immersed in a glass tank (T) containing water that was maintained at $25\text{--}28^\circ$ by recirculation from an adjoining cooled vessel (V) by means of a force pump (P) fixed in the tank. The pump circulated water at the rate of 0.8 l./min. and caused complete recirculation of the water in the tank every 20 min., and prevented a significant rise in temperature that could have resulted from the heating effect of the 1000 w lamp (X) used as a light-source and placed laterally to the tank at a distance of 20 cm. The remaining sides and the base of the tank were covered with aluminium foil to provide light-reflecting surfaces. An appropriate amount of $\text{Ba}^{14}\text{CO}_3$ (specific activity about 0.1 mc./mg.) was weighed into the flask G and $^{14}\text{CO}_2$ generated slowly by running in lactic acid (50%, v/v) from a small separating funnel (L). Approx. $100\text{ }\mu\text{C}$ of $^{14}\text{CO}_2$ was used in each experiment, the liberated gas being pumped into the flask containing the plant material and recirculated through the flask and around the closed systems by means of a diaphragm pump (D) in a small bell-jar; the total volume of diluent air was about 1500 ml. Plants were exposed for the required time (usually 3 hr.) with continuous illumination. At the end of the incubation, the clips B_1 and B_2 were opened and clip A was closed, so that excess of $^{14}\text{CO}_2$ in the system was pumped through a series of three traps containing a saturated solution of $\text{Ba}(\text{OH})_2$, and trapped as BaCO_3 . The incubation flask (F) was then removed for further examination.

Extraction and separation of terpenoids. The tissues were cut into small pieces and extracted with acetone (50 ml.) in an MSE homogenizer for 60 sec., the acetone was filtered off and the residue was re-extracted. The procedure was repeated until the final extract was colourless (usually three or four extractions were required). An equal volume of distilled water was added to the combined acetone extracts and the mixture exhaustively extracted three or four times with redistilled diethyl ether (50 ml. portions). The combined ether extracts were freed from acetone with water and then dried over anhydrous Na_2SO_4 (20 g./100 ml. of extract) for 30 min. The Na_2SO_4 was filtered off and washed with diethyl ether; the ethereal extract was evaporated to small volume at 30° under reduced pressure in a rotary evaporator, and quantitatively transferred to a small conical flask (25 ml.). Any residual water was removed by adding a few millilitres of ethanol and evaporating to dryness under N_2 . The dry lipid was weighed and then made to known volume for determination of chlorophyll or for radioassay.

Saponification and extraction of unsaponifiable matter. The method of Goodwin (1955) was used with the difference that the saponification was carried out at room temperature overnight and not with heating.

The sterols were removed from the unsaponifiable matter as their digitonides (Windaus & Welsch, 1909). They were purified by centrifuging at $600g$ and washing the precipitate twice with acetone-ethanol (1:1, v/v) and finally with diethyl ether until all yellow pigment was removed. The washings were combined with the supernatant and examined as 'sterol-free unsaponifiable material'. The precipitate was finally dried gently in a stream of N_2 over a steam bath and stored in a desiccator over CaCl_2 or P_2O_5 . The sterols were regenerated, when required, with pyridine (Schoenheimer & Dam, 1933).

Separation of terpenoids. The unsaponifiable components were first separated into crude fractions on columns of alumina (Brockmann grade 3) that had first been flushed with purified light petroleum (b.p. $40\text{--}60^\circ$) to remove impurities (Mercer, Davies & Goodwin, 1963). Elution was carried out with light petroleum containing increasing amounts of diethyl ether; details are given in Scheme 1. The crude fractions were then purified by thin-layer

Scheme 1. Separation of the unsaponifiable matter from seedlings. E/P, diethyl ether–light petroleum (% v/v).

techniques (see Davies, 1965) on plates of Kieselgel G, Kieselguhr G and alumina (all from E. Merck A.-G., Darmstadt, Germany).

Sterols were also identified by gas–liquid chromatography (Pye Argon chromatography) with argon as the mobile phase, kieselguhr as solid support and a silicone (Dow Corning QF-1) as liquid component. The columns (4ft. long $\times$ 4 mm. internal diam.) were packed from a preparation of 20g. of kieselguhr support mixed with 100 ml. of QF-1 (5%, v/v) in toluene. The columns were then developed at 219° with a gas flow of 43 ml./min., a detector voltage of 1500 v and the recording unit at sensitivity $\times$ 3. A scanning speed of 6 in./hr. was used and under these conditions all the sterols examined were completely eluted in less than 90 min.

β -Carotene was crystallized by the method of Goodwin (1958). Sterols were crystallized from either methanol containing a small amount of ethyl acetate or from aq. 80% (v/v) methanol by dissolving the solid in the minimum amount of hot solvent and allowing the solution to stand overnight in the cold room. The crystals were washed with ice-cold methanol, and, when radioactive, recrystallized to constant specific activity. Phytol was separated and detected by the method of Mercer & Goodwin (1962, 1963).

Quantitative determination of components. Chlorophyll was determined spectrophotometrically (Arnon, 1949), as were the various carotenoids (Davies, 1965); the sterols were also determined spectrophotometrically by the Liebermann–Burchard reaction (Davies, 1963). Measurements were made with either a Unicam SP.500 spectrophotometer or a Perkin–Elmer model 137 automatic spectrophotometer.

Tracer techniques. The ^{14}C -labelled compounds were counted either in a windowless 2π gas-flow proportional counter (Nuclear Measurements model PCC10A) with an argon+methane (9:1) gas mixture, the pulses being recorded on a Panax Unit 100C, or in a Packard Tri-Carb liquid-scintillation spectrometer (series 314E with Packard automatic control and automatic sample changer, model 500D). Radioactive paper chromatograms or thin-layer Neatan polymers were counted automatically in the Actigraph II instrument (Nuclear–Chicago model C1001A); the detector was a thin end-window (Micomil, 150 $\mu\text{g./cm.}^2$) gas-flow counter (Nuclear–Chicago model D47) with a butane+helium (1.3:98.7) gas mixture.

Radioautographs of paper chromatograms and thin-layer plates were obtained conventionally by exposing the paper or plate to fast X-ray film (Kodirex; Kodak Ltd.).

RESULTS

Monocotyledons. The incorporation of ^{14}C from $^{14}\text{CO}_2$ and $[2\text{-}^{14}\text{C}]$ mevalonic acid into various unsaponifiable components of etiolated maize illuminated for various times was compared. Etiolated seedlings (6–7 days old) were excised from their roots and continuously illuminated for a 12 hr., 24 hr., 36 hr., or 48 hr. period. In the carbon dioxide experiment they were exposed to $^{14}\text{CO}_2$ for the last 3 hr. of each period, and in the mevalonic acid experiment the exposure to $[2\text{-}^{14}\text{C}]$ mevalonate was for the last 6 hr. of each period. The results obtained with $^{14}\text{CO}_2$ are given in Table 1 and those with $[2\text{-}^{14}\text{C}]$ mevalonate in Table 2.

Three facts emerge from Table 1. Firstly, β -carotene and phytol are highly labelled after 3 hr. exposure at the 24 hr. stage of chloroplast development, whereas a similar exposure at the 36 and 48 hr. stage results in a much lower incorporation. It is clear that as the developing chloroplast matures, as evidenced by a decreased rate of chlorophyll synthesis, incorporation into the terpenoid components slows down; turnover of these components in mature chloroplasts is therefore also limited. Secondly, there is clearly a specific incorporation of $^{14}\text{CO}_2$ into the β -carotene and phytol and a failure to incorporate $^{14}\text{CO}_2$ significantly into steroids; e.g. some 4.5% of the total counts are in β -carotene, which normally represents about 0.2% of the total unsaponifiable matter, whereas insignificant counts are present in the sterols, which represent some 20% of the total unsaponifiable matter. Thirdly, a considerable amount of radioactivity appears in compounds not yet identified; e.g. at 24 hr. β -carotene

Table 1. *Incorporation of $^{14}\text{CO}_2$ into terpenoids of illuminated etiolated maize seedlings*Seedlings (2.5 g.) were exposed during the final 3 hr. of illumination to $100\mu\text{C}$ of $^{14}\text{CO}_2$ in the apparatus outlined in Fig. 1.

Total time of exposure (hr.)	Concn. of chlorophyll (mg./2.5 g.)	Percentage of ^{14}C available incorporated into unsaponifiable matter	Radioactivity in unsaponifiable matter (disintegrations/min.)			
			Total	β -Carotene	Phytol	Sterols
12	0.125	0.08	135 000 (6.0 mg.)	580	—	0
24	0.494	0.46	499 000 (5.2 mg.)	23 000	54 200	78
36	0.526	0.22	300 000 (8.8 mg.)	1310	3520	24
48	0.638	0.13	215 000 (12.9 mg.)	1310	1900	67

Table 2. *Incorporation of $[2-^{14}\text{C}]$ mevalonate into terpenoids of illuminated etiolated maize seedlings*Seedlings (2.5 g.) were exposed during the final 6 hr. of illumination to $10\mu\text{C}$ of DL- $[2-^{14}\text{C}]$ mevalonic acid.

Total time of exposure (hr.)	Percentage of ^{14}C available incorporated into unsaponifiable matter	Radioactivity in unsaponifiable matter (disintegrations/min.)			
		Total	β -Carotene	Phytol	Sterols
12	9.2	992 200 (13.3 mg.)	26 (10 $\mu\text{g.}$)	—	58 200 (2.6 mg.)
24	10.8	1 189 000 (14.5 mg.)	50 (21 $\mu\text{g.}$)	96	63 900 (3.1 mg.)
36	10.7	1 173 600 (14.8 mg.)	65 (24 $\mu\text{g.}$)	120	66 900 (3.5 mg.)
48	9.9	1 084 200 (13.9 mg.)	72	—	65 300 (3.4 mg.)

Table 3. *Incorporation of $^{14}\text{CO}_2$ into terpenoids of illuminated etiolated oat seedlings*Seedlings (3 g.) were exposed during the final 3 hr. of illumination to $100\mu\text{C}$ of $^{14}\text{CO}_2$ in the apparatus outlined in Fig. 1.

Total time of exposure (hr.)	Concn. of chlorophyll (mg./3 g.)	Percentage of ^{14}C available incorporated into unsaponifiable matter	Radioactivity in unsaponifiable matter (disintegrations/min.)			
			Total	β -Carotene	Phytol	Sterols
12	0.268	0.25	443 000 (14.2 mg.)	1400	1700	0
24	0.690	2.32	3 500 000 (15.6 mg.)	61 800	156 700	440
30	0.752	1.18	2 830 000 (14.8 mg.)	45 900	72 000	360
48	0.808	0.28	497 000 (10.2 mg.)	2100	3200	90
7 days*	—	0.24	413 000 (22.1 mg.)	1000	—	—

* Seedlings grown in continuous light for 7 days as control.

Table 4. *Incorporation of $[2-^{14}\text{C}]$ mevalonate into terpenoids of illuminated etiolated oat seedlings*Seedlings (3 g.) were exposed during the final 6 hr. of illumination to $10\mu\text{C}$ of DL- $[2-^{14}\text{C}]$ mevalonic acid.

Total time of exposure (hr.)	Percentage of ^{14}C available incorporated into unsaponifiable matter	Radioactivity in unsaponifiable matter (disintegrations/min.)					
		Total	β -Carotene	Phytol	Sterols	Squalene	β -Amyrin
6	5.25	462 000 (5.4 mg.)	0	40	186 000 (0.98 mg.)	1200	281 000
12	6.55	576 000 (6.3 mg.)	46	60	273 000	12 900	214 000
24	7.46	657 000 (7.2 mg.)	90	28	310 000 (1.8 mg.)	15 300	—
36	8.12	713 000 (7.9 mg.)	120*	55*	346 000 (2.1 mg.)	32 100*	270 000
48	8.53	749 000 (8.4 mg.)			331 000 (1.9 mg.)		282 000

* Counts in combined 36 hr. and 48 hr. fractions.

and phytol account for only some 15% of the total radioactivity incorporated into the unsaponifiable fraction.

With [2- ^{14}C]mevalonate the situation is reversed (Table 2). The incorporation is essentially independent of the state of development of the chloroplast; some 80% of the radioactivity is in the sterols and only very small amounts of radioactivity are in β -carotene and phytol. The small amount of radioactivity in β -carotene is real and the specific activity in all cases is about 2500 disintegrations/min./mg. compared with that of about 20000 disintegrations/min./mg. for the sterols; however, this does not take into account the very large endogenous pool of sterol present at the beginning of the experiment, which would make the specific activity of the newly synthesized sterol much greater. The radioactivity accounted for in known compounds is much greater with [2- ^{14}C]mevalonate than with $^{14}\text{CO}_2$.

Similar experiments with oats confirmed that ^{14}C from $^{14}\text{CO}_2$ is much more effectively incorporated into β -carotene and phytol than into the plant sterols, and that incorporation reaches a maximum after 24–30 hr. and falls rapidly thereafter (Table 3). The very low turnover of $^{14}\text{CO}_2$ in mature chloroplast terpenoids is reflected in the results on 7-day seedlings grown continuously in the light in which the total incorporation into β -carotene is some 60-fold less than into etiolated seedlings illuminated for 24 hr. With [2- ^{14}C]mevalonate the sterols are very strongly labelled (Table 4), as they are in maize (Table 2). In this experiment β -amyrin and squalene were also examined and they were also heavily labelled; β -carotene was essentially un-

labelled. To emphasize the effect of pre-illumination on the incorporation of ^{14}C from $^{14}\text{CO}_2$ and [2- ^{14}C]mevalonate into unsaponifiable material some of the data in Tables 3 and 4 are collected in Fig. 2.

Dicotyledons. Experiments similar to those described in the previous section were carried out on peas and French beans. With peas (Tables 5 and 6) the pattern is the same as that in maize and oats in the sense that ^{14}C from $^{14}\text{CO}_2$ is relatively much more effectively incorporated into β -carotene and phytol than into sterols. There are, however, some

Fig. 2. Effect of pre-illumination time on the incorporation of $^{14}\text{CO}_2$ and [2- ^{14}C]mevalonate into illuminated etiolated oat seedlings. Experimental conditions were as outlined in Tables 3 and 4. □, Concn. of chlorophyll (mg./3g. of seedlings); ○, radioactivity (disintegrations/min.) in β -carotene from $^{14}\text{CO}_2$; ●, radioactivity (disintegrations/min.) in sterols from [2- ^{14}C]mevalonate.

Table 5. Incorporation of $^{14}\text{CO}_2$ into terpenoids of illuminated etiolated pea seedlings

Seedlings (10g.) were exposed during the final 3 hr. of illumination to $100\mu\text{C}$ of $^{14}\text{CO}_2$ in the apparatus outlined in Fig. 1.

Total time of exposure (hr.)	Radioactivity in unsaponifiable matter (disintegrations/min.)			
	Total	β -Carotene	Phytol	Sterols
6	18200 (22.5 mg.)	5400	11300	0 (5.9 mg.)
24	440000 (26.7 mg.)	26000	48800	—
72	1990000 (26.1 mg.)	221000	174000	56 (8.1 mg.)
100	1470000 (20.4 mg.)	170000	115000	140 (7.8 mg.)
7 days green	1760000 (31.8 mg.)	136000	97300	—

Table 6. Incorporation of [2- ^{14}C]mevalonate into terpenoids of illuminated pea seedlings

Seedlings (10g.) were exposed during the final 6 hr. of illumination to $10\mu\text{C}$ of DL-[2- ^{14}C]mevalonic acid.

Total time of exposure (hr.)	Concn. of chlorophyll (mg./10g.)	Radioactivity in unsaponifiable matter (disintegrations/min.)			
		Total	β -Carotene	Sterols	Squalene
6	0.005	293000 (13.4 mg.)	0 (5.6 $\mu\text{g.}$)	36000 (2.45 mg.)	—
30	0.121	322000 (11.2 mg.)	—	104000 (5.68 mg.)	17800
60	0.940	463000 (17.8 mg.)	86 (14.4 $\mu\text{g.}$)	126000 (7.10 mg.)	—
96	1.334	635000 (24.2 mg.)	315 (21.0 $\mu\text{g.}$)	153000 (8.62 mg.)	275000

Table 7. *Incorporation of $^{14}\text{CO}_2$ into terpenoids of mature lettuce heart leaves during chloroplast development*

Leaves (10g.) were exposed during the final 3hr. of illumination to $100\mu\text{C}$ of $^{14}\text{CO}_2$ in the apparatus outlined in Fig. 1.

Total time of exposure (hr.)	Radioactivity in unsaponifiable matter (disintegrations/min.)		
	Total	β -Carotene	Sterols
12	434 000 (8.5 mg.)	7 000 (8.2 $\mu\text{g.}$)	235 (1.7 mg.)
24	1 200 000 (7.8 mg.)	22 700 (12.4 $\mu\text{g.}$)	—
30	1 990 000 (17.3 mg.)	31 200 (17.7 $\mu\text{g.}$)	947 (2.1 mg.)
48	1 656 000 (19.0 mg.)	6 700 (4.6 $\mu\text{g.}$)	1 420 (2.5 mg.)
Mature outer leaf (20 g.)	574 000 (26.2 mg.)	7 900 (28.7 $\mu\text{g.}$)	1 660 (9.2 mg.)

Table 8. *Incorporation of $[2\text{-}^{14}\text{C}]$ mevalonate into terpenoids of mature lettuce heart leaves during chloroplast development*

Leaves (10g.) were exposed during the final 6hr. of illumination to $10\mu\text{C}$ of DL- $[2\text{-}^{14}\text{C}]$ mevalonate.

Total time of exposure (hr.)	Concn. of chlorophyll (mg./10g.)	Radioactivity in unsaponifiable matter (disintegrations/min.)			
		Total	β -Carotene	Sterols	Squalene
6	0.074	174 000 (15.0 mg.)	0	65 100 (2.1 mg.)	2760
24	0.410	196 000 (15.9 mg.)	0 (10.4 $\mu\text{g.}$)	67 100 (2.6 mg.)	1080
32*	—	139 000 (13.6 mg.)	128 (12.8 $\mu\text{g.}$)	88 900 (3.7 mg.)	1650
56	0.491	257 000 (20.8 mg.)	43 (14.4 $\mu\text{g.}$)	54 300 (2.2 mg.)	3030
72	0.563	214 000 (16.5 mg.)	177 (13.6 $\mu\text{g.}$)	69 900 (2.9 mg.)	1970
Mature outer green leaf	—	107 000 (8.9 mg.)	0 (27.8 $\mu\text{g.}$)	—	3900

* 15g. of tissue was used, but the values are related to 10g. of tissue.

differences: first, the uptake of ^{14}C from $[2\text{-}^{14}\text{C}]$ -mevalonate is better in oats and maize (maximum uptake 745 000 disintegrations/min. in unsaponifiable matter from 3g. of oat tissue) than in peas (636 000 disintegrations/min. in unsaponifiable matter from 10g. of pea tissue); secondly, full development of the pea chloroplast, as measured by chlorophyll concentrations, takes some 60–96hr. compared with 24hr. for oats and maize; thus maximum uptake of ^{14}C from $^{14}\text{CO}_2$ into β -carotene and phytol does not occur until after 72hr. and is still considerable after 100hr.; thirdly, mature leaves, 7 days old, grown in continuous light still actively incorporate label into β -carotene and phytol. Very similar results were observed with French beans and are not recorded here.

Experiments were also carried out on lettuce heart leaves; these represent mature etiolated tissues whereas all the previous experiments were carried out on immature tissue. The mature lettuces were maintained in water culture until required; the heart leaves were then exposed to $^{14}\text{CO}_2$ or $[2\text{-}^{14}\text{C}]$ mevalonic acid under standard conditions. The results obtained show that, as they green up, mature etiolated tissues incorporate ^{14}C from $^{14}\text{CO}_2$ specifically into β -carotene compared with sterols and that the incorporation was maximal

after 24hr. (Table 7); radioautographs indicated heavy incorporation into the phytol and xanthophyll fractions. With $[2\text{-}^{14}\text{C}]$ mevalonate, sterols and squalene are strongly labelled, whereas little or no radioactivity is found in β -carotene (Table 8); radioautographs also showed little radioactivity in the phytol and xanthophyll areas of the chromatogram. Further, the degree of incorporation is independent of the degree of etiolation and is the same in mature green leaves.

DISCUSSION

In all the plants examined β -carotene and phytol (from chlorophyll), two typical chloroplast terpenoids, are strongly labelled from $^{14}\text{CO}_2$ during chloroplast development, whereas the sterols present, some of which are located in the chloroplast (Davies, 1963), are insignificantly labelled; the degree of labelling is dependent on the state of chloroplast development. These observations indicate that chloroplast terpenoids are synthesized in the chloroplast and that sterols are synthesized outside the chloroplast. If this is so then, as the biosynthetic pathways of all terpenoids pass through a number of common steps, it must be considered that common enzymes, e.g. those catalysing the steps from meva-

lonate to farnesyl pyrophosphate (see Goodwin, 1965), are present both inside and outside the chloroplast, whereas certain specific enzymes are located entirely or to a very great extent within specific organelles; e.g. squalene synthetase (2 farnesyl pyrophosphate $\rightarrow$ squalene) is located outside the chloroplast and phytoene synthetase (2 geranylgeranyl pyrophosphate $\rightarrow$ phytoene) inside the chloroplast. In support of this view, Rogers, Shah & Goodwin (1965) have demonstrated the presence of mevalonate kinase, the first step in mevalonate metabolism, both inside and outside chloroplasts. In addition to enzyme segregation there must also exist a mechanism that prevents one or all of the common substrates leaking from the chloroplast during the active synthesis of chloroplast terpenoids. As a working hypothesis we propose that the limiting membrane of the chloroplast is impermeable or only slightly permeable to mevalonate, the first biosynthetic intermediate specific to terpenoids. It may be argued that the membrane is impermeable to all such intermediates and it is only sucrose, which would not be produced in great amounts in 3 hr. exposure to carbon dioxide in developing chloroplasts, which passes into the cytoplasm. However, in unpublished work in our Laboratory D. R. Threlfall & W. T. Griffiths have found the same distribution of label between β -carotene and sterols after exposure of seedlings to $^{14}\text{CO}_2$ for as long as 24 hr. and we observed the same distribution in mature lettuce leaves exposed for 3 hr. (Table 7). Rogers *et al.* (1965) have demonstrated the essential impermeability of non-aqueously prepared chloroplasts to mevalonic acid, but the present experiments *in vivo* do indicate a slight but real incorporation into chloroplast carotenoids, especially in dicotyledons. However, even in these plants the incorporation is different from the situation with carbon dioxide; it is relatively much less than into sterols, although this may not appear so at first sight. If the most unfavourable case, the 96 hr. results in Table 6, is considered, the specific activity based on total amounts present in disintegrations/min./mg. is about 15 000 for β -carotene and about 18 000 for sterols; if the values are based on the increment synthesized during the 6 hr. exposure then the corresponding values are about 300 000 and 600 000 respectively. Direct evidence for the existence of a large unlabelled pool of endogenous sterols has come from unpublished work by W. T. Griffiths & D. R. Threlfall, who have found that, in similar experiments to those described in this paper, the sterol esters, which represent about 4% of the total sterols, contain over 80% of the radioactivity after exposure of maize leaves to $[2-^{14}\text{C}]$ mevalonate.

In the monocotyledons maize and oats, the incorporation of ^{14}C from $^{14}\text{CO}_2$ into β -carotene and phytol is maximal in seedlings exposed to light for 24 hr., at which time, as indicated by chlorophyll content, there is maximal rate of chloroplast development. The very sharp decrease in incorporation in monocotyledonous seedlings with mature chloroplasts indicates a second mechanism that diverts carbon dioxide away from terpenoid synthesis when these compounds are no longer required; further, there must be very little turnover of these pigments. This latter observation confirms those of Perkins & Roberts (1962) on the low chlorophyll turnover in monocotyledons. The 'cut-out' mechanism might be due to the disappearance of key enzymes, because Falk, Appleby & Porra (1959) found that certain key enzymes involved in chlorophyll biosynthesis that are present in homogenates of young oat seedlings are absent from mature leaves. However, in green fruit, such as tomatoes, chloroplasts change during ontogeny into chromoplasts in which chlorophyll (with its phytol residue) disappears and carotenoids, in this case lycopene, are very actively synthesized.

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