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Incorporation of DL-[2-^{14}C]Mevalonic Acid Lactone into β-Carotene and the Phytol Side Chain of Chlorophyll in Cotyledons of Four Species of Pine Seedlings

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1. The incorporation of DL-[2-^{14}C]mevalonic acid lactone into β-carotene and the phytol side chain of chlorophyll has been investigated in cotyledons of four species of pine seedlings (*Pinus silvestris*, *P. contorta*, *P. radiata* and *P. jeffrei*) grown in darkness and in light. 2. The relative incorporation of label into β-carotene and the phytol side chain of chlorophyll is similar to that observed in experiments on monocotyledons and dicotyledons. 3. The relative incorporation of $^{14}CO_2$ into β-carotene and phytol is much higher than the incorporation of [2-^{14}C]mevalonic acid.

It is generally known that MVA‡ is an intermediate in the biosynthesis of all terpenoids and it is converted into these compounds only (Popják & Cornforth, 1960). Comparative studies on the incorporation of [2-^{14}C]MVA and $^{14}CO_2$ into various terpenoids in plants led to the conclusion that exogenous [2-^{14}C]MVA is effectively incorporated into all terpenoids only in very primitive organisms. In angiosperms it is incorporated only into those that are synthesized outside the chloroplasts (squalene, sterols, ubiquinone). On the other hand, $^{14}CO_2$ is much more easily incorporated into terpenoids that are formed inside the chloroplasts (see Goodwin, 1965, 1967). However, in all these experiments a small amount of [2-^{14}C]MVA is converted into β-carotene even when this pigment is rigorously purified (Treharne, Mercer & Goodwin, 1966).

To explain these facts the hypothesis has been proposed (Goodwin, 1958; Goodwin & Mercer, 1963) that there exist two sites for biosynthesis of terpenoids in plant cells, one inside and the other outside the chloroplasts. The comparative impermeability of chloroplast membranes to some metabolites, among them MVA, plays an important part in the separation of these two sites of synthesis. Thus exogenous [2-^{14}C]MVA and $^{14}CO_2$ are converted into terpenoids in two segregated biosynthetic systems.

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‡ Abbreviation: MVA, mevalonic acid.

A comparison of the incorporation of [2-^{14}C]MVA lactone into β-carotene and the phytol side chain of chlorophyll in some species of pine seedlings grown in light and in darkness was the main aim of the experiments described below. Pine seedlings are particularly noteworthy in this respect because they are able to synthesize chlorophylls and carotenoids in the dark (Schmidt, 1924; Bogorad, 1950; Wieckowski & Goodwin, 1966).

MATERIALS AND METHODS

Plant cultivation. The experiments were carried out on the cotyledons of four species of pine seedlings: *Pinus silvestris*, *P. contorta*, *P. radiata* and *P. jeffrei*.

Seeds, after soaking in water for 24hr., were put into boxes with soil (mixture of compost with sand, 1:1). They were germinated and grown in these boxes in either dark or light (temperature 29°). The seedlings growing in light were continuously illuminated by fluorescent tubes (about 300 lux). All manipulations connected with the cultivation of seedlings in darkness were carried out in neutral green light (Withrow & Price, 1957).

Bean seedlings (*Phaseolus vulgaris* var. French) were cultivated in this same manner and in the same conditions.

Introduction of label into plants. DL-[2-^{14}C]MVA lactone in water solution was introduced into the plants in two different ways. In most of the experiments the cotyledons of pine seedlings or primary leaves of bean were cut into small pieces and immersed in [2-^{14}C]MVA solution (8μc/about 400mg. fresh wt.). In some experiments the [2-^{14}C]MVA was introduced into plants by their stems, i.e. seedlings excised from their roots were immersed in [2-^{14}C]MVA solution.

$^{14}CO_2$ was liberated from $Ba^{14}CO_3$ by dilute lactic acid. The experiments were carried out in a special glass chamber (Treharne *et al.* 1966). Seedlings excised from their roots

were immersed in water by their stems. Seedlings were exposed to labelled substrate for 6hr.

Purification and estimation of β-carotene and phytol. Lipid material was extracted into ether according to our standard procedures (Treharne *et al.* 1966). The ether solution, after drying over anhydrous Na_2SO_4, was made up to constant volume and a small volume was taken for estimation of radioactivity (total radioactivity of lipid fraction). The ether was then evaporated *in vacuo* and the residue dissolved in light petroleum (b.p. 40–60°) (A.R., dehydrated with sodium metal and distilled). Pigments were separated chromatographically on a Hyflo Super-Cel column (about 15cm. × 1cm.). The chromatogram was developed with light petroleum containing increasing amounts of ether. The carotene and chlorophyll fractions were kept for further purification.

The carotene fraction was concentrated and passed through a chromatographic column of MgO. β-Carotene was separated from α-carotene, phytoene, phytofluene and squalene. Light petroleum with increasing amount of acetone was used for the development of the column. The β-carotene fraction was evaporated to dryness and the pigment dissolved in a known volume of light petroleum. The E_{450} value of the solution was measured with a Unicam SP.500 spectrophotometer. The amount of pigment present was calculated from $E^{1\%}_{1cm.}$ 2505. From the same sample a known volume was put on a thin-layer chromatographic plate (silica gel G; E. Merck A.-G.). Light petroleum was used for the development of the chromatogram. The β-carotene zone was eluted for the radioactivity assay.

Chlorophylls were separated from pheophytins and chlorophyllides on a Hyflo Super-Cel column. After removal of the solvent the pigments were dissolved in ether and made up to known volume. The E_{662} and E_{644} values were measured. The amounts of chlorophylls *a* and *b* were then calculated from the equation of Smith & Benitez (1955). The amount of phytol present was calculated from the amounts of chlorophylls *a* and *b*. After spectrophotometric estimation, the ether was removed *in vacuo* and the pigments were dissolved in ethanol and saponified (Goodwin, 1955). Phytol was extracted with ether and after drying over anhydrous Na_2SO_4 the ether was evaporated *in vacuo* and the residue dissolved in light petroleum. Further purification was carried out chromatographically on alumina (Woelm; neutral, Brockmann grade III) and on thin-layer plates (silica gel G). Phytol was eluted from the column with 5–7% (v/v) ether in light petroleum. Chloroform was used for the development of the chromatogram on the plate (Treharne, 1964). The purified phytol was eluted from the plate for assay of radioactivity.

The radioactivities of the purified compounds were measured with a Packard Tri-Carb scintillation spectrometer.

Examination of β-carotene and phytol for radioactive purity. Labelled β-carotene purified by the methods described above was crystallized (Goodwin, 1958). After each crystallization the crystals were washed several times with ice-cold methanol before being assayed for radioactivity. The results are presented in Table 1. It shows that the β-carotene purified on two chromatographic columns and by thin-layer chromatography is free from radioactive impurities.

The radioactive purity of phytol was checked on radioautographs. The spots coincided exactly with authentic phytol and when the chromatograms were scanned (Nuclear–Chicago Actigraph Scanner) the radioactivity in the phytol zone was distributed very symmetrically.

Table 1. *Specific radioactivity of β-carotene after crystallization*

Preparation of purified non-crystalline β-carotene was by a combination of column and thin-layer chromatography (see the Materials and Methods section).

	10^{-4} × Sp. radioactivity (counts/min./mg.)
Before crystallization	2·87
After first crystallization	2·63
After second crystallization	2·88

RESULTS

Table 2 shows that the amount of label that is assimilated by 100 pine seedlings under standard conditions is very dependent on the species and varies between 3×10^6 and 44×10^6 counts/min. In general, seedlings grown in light show higher specific radioactivities than those grown in darkness, but in spite of this in all cases 0·1–0·4% and 0·5–3% of assimilated label is incorporated into β-carotene and phytol respectively. After 6 days' cultivation in darkness these percentages are generally smaller. Although it is difficult to assess the significance of specific radioactivity measurements in a system where the rate of synthesis of a compound has not been determined, the specific radioactivities have been calculated on the basis of total carotene and phytol present. They vary considerably with the species, *P. silvestris*, which takes up rather little MVA, giving the highest values.

The radioactivities of whole lipid fraction, β-carotene and phytol depend on the method of introduction of [2-^{14}C]MVA into the plants, and on the concentration of this labelled precursor (Table 3). [2-^{14}C]MVA introduced into leaves cut into small pieces is about seven times as effectively incorporated into various terpenoid compounds as when it is introduced by the stem. However, the percentage of the ^{14}C that is incorporated into β-carotene and phytol is in all cases more or less the same: about 0·3% is incorporated into β-carotene and about 1% into the phytol side chain of chlorophyll.

Table 4 shows that seedlings of *P. jeffrei* grown in darkness and then illuminated for 6hr. in the presence of $^{14}CO_2$ incorporated ^{14}C less effectively than those grown in continuous light. The specific radioactivity of β-carotene is also lower. But in both cases more or less the same percentage of ^{14}C

Table 2. *Incorporation of* [2-^{14}C]*MVA into pine seedlings*

Total radioactivity of lipid fraction and specific radioactivities of β-carotene and the phytol side chain of chlorophyll in seedlings incubated with [2-^{14}C]MVA in darkness (d) and in light (l) were measured. For details of germination see the Materials and Methods section.

		2 days after germination				6 days after germination					
			β-Carotene		Phytol		β-Carotene		Phytol		
		$10^{-6}\times$ Total radioactivity of lipid fraction* (counts/min./100 plants)	$10^{-5}\times$ Sp. radioactivity (counts/min./mg.)	Radio-activity (% of total)	$10^{-5}\times$ Sp. radioactivity (counts/min./mg.)	Radio-activity (% of total)	$10^{-6}\times$ Total radioactivity of lipid fraction* (counts/min./100 plants)	$10^{-5}\times$ Sp. radioactivity	Radio-activity (% of total)	$10^{-5}\times$ Sp. radioactivity (counts/min./mg.)	Radio-activity (% of total)
P. jeffrei	d	42·2	2·48	0·07	4·14	0·81	54·2	3·26	0·14	3·83	0·81
	l	44·3	5·24	0·21	4·22	1·61					
P. silvestris	d	5·33	17·8	0·24	33·8	1·84	6·10	10·95	0·19	9·66	0·62
	l	7·76	17·3	0·13	28·2	2·43					
P. radiata	d	10·22	6·61	0·38	7·55	1·58	3·70	0·32	0·07	0·68	0·69
	l	15·4	6·21	0·32	9·76	3·07					
P. contorta	d	3·37	5·00	0·13	5·35	0·50	2·78	2·43	0·05	7·30	0·58
	l	3·14	11·96	0·29	6·71	0·89					

* Essentially the total incorporation into terpenoids.

is found in both compounds, and these are much greater than when [2-^{14}C]MVA is the labelled substrate.

The differences between the incorporation of $^{14}CO_2$ and [2-^{14}C]MVA into β-carotene and into the sterol fraction (Table 4) is very marked; when [2-^{14}C]MVA is introduced only 0·3% is incorporated into β-carotene and when $^{14}CO_2$ about 7%. When the comparison is between β-carotene and total lipid the difference is less marked, but this is because $^{14}CO_2$ is also actively incorporated into neutral lipid.

DISCUSSION

These results confirm our earlier work on the incorporation of [2-^{14}C]MVA and $^{14}CO_2$ into β-carotene and the phytol side chain of other monocotyledons and dicotyledons (Treharne *et al.* 1966). That is, in the light $^{14}CO_2$ is effectively incorporated into the plastid terpenoids and less effectively incorporated into the sterols, whereas with [2-^{14}C]-MVA the situation is reversed. These observations have been interpreted as indicating a means by which regulation of terpenoid synthesis is obtained by a combination of enzyme segregation and specific membrane permeability of the chloroplast. That is, although the chloroplast can share certain enzymes of the terpenoid biosynthetic sequence, the chloroplast is comparatively impermeable to the first compound specific to terpenoid biosynthesis, MVA. It followed from this that failure of plastids to form pigments in the dark was due to failure of MVA synthesized extraplastidically to enter the plastid. If this were so then, in the cotyledons of pine seedlings that synthesize plastid terpenoids in the dark, the membrane might be expected to be permeable to MVA, so that terpenoid synthesis could proceed. However, the general pattern is very little different from that in plants that cannot synthesize plastid terpenoids in the dark. It may be that in this case other factors are more important: for example the enzymes concerned may be present in high concentration in the plastids produced in the dark by pine cotyledons. Certainly in other tissues, e.g. in bean and maize, it appears that synthesis of enzymes is necessary before terpenoid synthesis can take place. There is always a time lag before synthesis begins when seedlings grown in the dark are brought into the light. This lag can be overcome if the dark-grown seedlings are given a short exposure to red light 24–48 hr. before being brought out into the light. This is a phytochrome effect, and after the exposure the plastids swell to the size of chloroplasts and increase their content of protein (enzymes), so that when these seedlings are brought into the light terpenoid synthesis begins

Table 3. *Effects of the method of introduction of* [2-^{14}C]*MVA and its concentration on the radioactivities of total lipid fraction, β-carotene and phytol in etiolated bean plants*

Six-day-old plants were illuminated (light-intensity 600 lux) for 24 hr., during the last 6 hr. in the presence of labelled substrate. The temperature was 29°. In method I six leaves cut into small pieces were immersed in [2-^{14}C]MVA solution (8 μc in 2 ml. of water). In method II three seedlings excised from their roots were immersed by their stems in [2-^{14}C]MVA solution (8 μc in 4 ml. of water). In method III three seedlings excised from their roots were immersed by their stems in [2-^{14}C]MVA solution (2 μc in 4 ml. of water).

		β-Carotene			Phytol		
Method	10^{-5} × Total radioactivity of lipid fraction (counts/min.)	10^{-3} × Radioactivity (counts/min.)	Radioactivity (% of total)	10^{-4} × Sp. radioactivity (counts/min./mg.)	10^{-3} × Radioactivity (counts/min.)	Radioactivity (% of total)	10^{-4} × Sp. radioactivity (counts/min./mg.)
I	120·2	31·2	0·26	51·9	91·5	0·76	46·9
II	16·5	3·26	0·20	4·25	17·5	1·06	7·11
III	4·99	2·73	0·55	3·31	8·56	1·72	3·23

Table 4. *Comparison of incorporation of* $^{14}CO_2$ *and* [2-^{14}C]*MVA into sterols and β-carotene in cotyledons of* P. jeffrei

Seedlings were germinated for 3 days in either darkness or light and then transferred to light for 6 hr. in the presence of labelled substrate. The light intensity was about 600 lux. The temperature was 29°. Results are expressed as radioactivity incorporated per ten plants.

	10^{-4} × Radioactivity in 'crude' sterols (counts/min.)	10^{-3} × Radioactivity in β-carotene (counts/min.)	% of sterol radioactivity in β-carotene
$^{14}CO_2$ (60 μc)			
Light→light	27·4	20·5	7·47
Dark→light	8·45	5·74	6·78
[2-^{14}C]MVA (8 μc)			
Light→light	351	9·5	0·27
Dark→light	465	10·7	0·23

immediately (Mego & Jagendorf, 1961; Henshall & Goodwin, 1964). In pine cotyledons presumably the enzymes are present in sufficient quantity, but in this case light is not required to trigger off the synthesis of terpenoids within the organelle.

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