The Subcellular Distribution and Biosynthesis of Castaprenols and Plastoquinone in the Leaves of *Aesculus hippocastanum*

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(Received 2 February 1967)

Intact chloroplasts and cell walls were prepared from horse-chestnut leaves that had previously metabolized [2-¹⁴C]mevalonate. The bulk of the castaprenols and plastoquinone-9 was found within the chloroplasts. The remaining portion of the castaprenols was associated with the cell-wall preparation whereas that of the plastoquinone-9 was probably localized in the soluble fraction of the plant cell. The ¹⁴C content of these compounds of different cell fractions indicated the presence of polyisoprenoid-synthesizing activity both inside and outside the chloroplasts. This was confirmed by the relative incorporation of ¹⁴C when ultrasonically treated and intact chloroplasts were incubated with [2-¹⁴C]mevalonate. As the leaves aged (on the tree) an increase in extraplastidic castaprenols and plastoquinone-9, together with associated synthesizing activities, was observed.

Polyisoprenoid alcohols of up to 65 carbon atoms in length have been identified in the leaf tissue of many angiosperms (Wellburn & Hemming, 1966a). All isolated alcohols have the general formula (I) and usually occur naturally as mixtures of cogeners. Solanesol is the only natural all-*trans*-polyprenol so far described. It was first isolated from tobacco leaves and was shown to be all-*trans*-nonaprenol (*n* = 9) by Erickson, Shunk, Trenner, Arison & Folkers (1959) and by Kofler *et al.* (1959). Those alcohols from the leaves of *Aesculus hippocastanum* (horse chestnut), *Ficus elastica* (decorative rubber plant), *Betula verrucosa* (birch) and *Hevea brasiliensis* (rubber plant) have been fully characterized (Wellburn, Stevenson, Hemming & Morton, 1967; Stone, Wellburn, Hemming & Pennock, 1967; Wellburn & Hemming, 1966c; Dunphy, Kerr, Pennock, Whittle & Feeney, 1967). They have all been shown to exist naturally as mixtures of alcohols ranging from 50 to 65 carbon atoms in length and have been termed the castaprenols-10 to -13, ficaprenols-10 to -13 and betulaprenols-10 to -13 respectively.

In contrast with solanesol, where the protons on the double bond are solely in the *trans* position relative to the methyl groups, only three of the internal double bonds of the castaprenols, ficaprenols etc. are in the *trans* configuration. As the exact location of the isoprene units having the *cis* configuration within the isoprenoid chain has not

been established, it is not yet certain if the castaprenol, ficaprenol, betulaprenol and heveaprenol that correspond in chain length are stereochemically identical with each other. However, if in each prenol the three *trans* units are adjacent to the ω -terminal unit, it seems likely that, during biosynthesis, isoprene units with the *cis* configuration are added in turn to an all-*trans* precursor such as all-*trans*-geranylgeranyl pyrophosphate.

Mixtures of similar isoprenoid alcohols have also been isolated from the non-photosynthetic tissues of angiosperms. In birch wood, Lindgren (1965) has indicated the presence of betulaprenols-6 to -9. Subsequent isolation has shown them each to contain one less internal *trans*-isoprene residue than the corresponding betulaprenols-10 to -13 from birch leaves (Wellburn & Hemming, 1966c).

The quantitative distribution of isoprenoid alcohols in plant tissue has not been extensively investigated. Stevenson, Hemming & Morton (1963) studied the distribution of solanesol in various subfractions from tobacco-leaf homogenates and concluded that solanesol, like plastoquinone, is concentrated in the chloroplasts of the leaf. Preliminary experiments with horse-chestnut leaves

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have shown that the bulk of the castaprenols may also be recovered from the chloroplasts. After chloroplasts prepared by differential centrifugation had been treated with ultrasound, preparations of lamellae and so-called osmiophilic globules were obtained by using the method of Bailey & Whyborn (1963). Analysis of these fractions showed that the bulk of the seasonal increase of castaprenols within horse-chestnut leaves may be accounted for by the increase in castaprenol content of the osmiophilic-globule fraction (Wellburn & Hemming, 1966b). These results were substantiated the following year by similar analyses of fragments prepared from intact chloroplasts obtained by the Leech density-gradient method (Wellburn, 1966).

The following describes the distribution of the castaprenols, together with plastoquinone, in density-gradient-prepared chloroplasts and in cell-wall preparations from horse-chestnut leaves that had previously been allowed to metabolize [2-¹⁴C]-mevalonate.

METHODS AND MATERIALS

Expt. 1. Small branches of *Aesculus hippocastanum* bearing leaves were collected, from the same tree, at 16, 20 and 24 weeks after terminal-bud unfolding. To minimize chloroplast fragmentation by starch grains during centrifugation, the small branches were placed in a CO₂-free glass-walled illuminated incubator for 3 days. During this period DL-[2-¹⁴C]mevalonic acid (10 μ C, 2 μ moles) was incorporated via the stem of the 20- and 24-week-old branches from a small reservoir in which the ends of the stems were placed. The transpirational uptake of mevalonic acid was facilitated by efficient circulation of CO₂-free air through the incubator.

Chloroplasts with intact membranes were prepared from the first and second pairs of leaves above each annual ring by the method of Leech (1964). The debris from the chloroplast preparations was employed as the starting material for the preparation of purified cell walls by using a modified form of the method of Kivilaan, Beaman & Bandurski (1959). The debris (about 20 g.) was homogenized in the liquidizer of a Kenwood Chef for 10 min. at low speed, together with glycerol (180 ml.) and glass beads (37 g., 200 μ diam.; Minnesota Mining and Manufacturing Co., St Paul, Minn., U.S.A.). The homogenate was poured into an excess of water (about 5 l.) and was permitted to stand for 0.5 hr. Those particles that float to the surface form the best cell-wall preparation (P. D. Phethean, unpublished work). Accordingly, the upper layer was decanted into a coarse-porosity sintered-glass funnel (150 ml.) containing a filter bed of glass beads. Filtration, with slight suction, was accompanied by continual agitation to prevent mat formation. The filter bed was resuspended in glycerol (50 ml.) and the whole sequence was repeated. The cell-wall preparation was washed repeatedly with water and the beads forming the final filter bed were removed by centrifugation at 500 g for 5 min. The preparation was filtered on a fine-porosity sintered-glass funnel and was finally freeze-dried, together with a similar portion of original leaves, before the extraction of each.

Expt. 2. On each of two occasions chloroplast suspensions were prepared from leaves approx. 18 weeks old by the method of Leech (1964) and equivalent portions of each suspension were precipitated by centrifugation. Each portion was resuspended in cold 0.15 M-Na₂HPO₄-KH₂PO₄ buffer, pH 7.3, in 0.3 M-sucrose (20 ml.) containing DL-[2-¹⁴C]mevalonic acid (2 μ C, 0.4 μ mole), together with 0.02 M-ATP (1.5 ml.) and 0.1 M-MgCl₂ (2 ml.) in phosphate buffer-sucrose medium and neutralized 0.1 M-cysteine hydrochloride (1.5 ml.), all made up to 30 ml. with phosphate buffer-sucrose medium. One-half of each preparation was treated three times for 20 sec. each with an MSE 60 W ultrasonic disintegrator having a cooled titanium probe with an end ratio 3.6:1. The slight fall of pH on rupture of the chloroplast envelope was counteracted by the appropriate use of a phosphate buffer-sucrose medium of slightly higher pH. Each suspension, either of whole chloroplasts or of chloroplasts treated with ultrasound, was incubated in a closed flask at 30° for 3 hr. by using a photosynthetic Warburg apparatus with continuous agitation and illumination.

Lipid extraction. The lipid of preparations of chloroplasts was extracted by stirring with an excess of 80% (v/v) acetone. Dried whole leaves and cell-wall preparations were extracted three times with acetone (about 2 ml./g. of tissue) assisted by maceration with a small Ultra-Turrax homogenizer. The acetone extracts were filtered through a sintered-glass funnel under reduced pressure and were extracted three times with freshly distilled diethyl ether. The ethereal extracts were washed with water and dried (Na₂SO₄). The ether was then evaporated and the residues were blown to dryness under N₂.

Assay of lipid material. The lipid extracts were dissolved in light petroleum (b.p. 40–60°) and were chromatographed on columns (5 g.) of acid-washed alumina (Brockmann grade III). Plastoquinone, eluted in 2% (v/v) diethyl ether in light petroleum (b.p. 40–60°), was assayed in ethanolic solution by determination of the $\Delta E_{1}^{1\%}$ at 254 m μ with NaBH₄ (Crane, Lester, Widmer & Hatefi, 1959). Estimation of the castaprenols, eluted by 8% (v/v) diethyl ether in light petroleum (b.p. 40–60°), depended on comparing spot size against a range of spots of standard amounts of authentic material on thin layers of silica gel (275 μ thick) as suggested by Truter (1963). The sterols were estimated by using a modified form of the method of Zlatkis, Zak & Boyle (1953) as described by Leffler & McDougald (1963). Total chlorophyll, finally eluted by 5% (v/v) methanol in diethyl ether, was determined by the method of Vishniac (1957).

Assay of 'cellulose'. The method suggested by Monier & Williams (1921) for the hydrolysis of cell-wall fractions and samples of leaf tissue was adopted. Simultaneous estimation of the test (hydrolysed), blank (non-hydrolysed) and standard solutions was carried out in triplicate by using the combined methods of Somogyi (1945) and Nelson (1944) for the determination of reducing sugar. To minimize errors arising from a high concentration of dissolved O₂, all estimation tubes were placed in boiling water for 15 min. before estimation; Somogyi reagent (2 ml.) was added and boiling continued for a further 40 min. After cooling, Nelson's arsenomolybdate reagent (1 ml.) was added and E_{650} was determined after 1 min. in a Unicam SP.600 spectrophotometer. A standard curve of E_{650} value against concentration of reducing sugar was constructed from the determinations on standard solutions of glucose conducted

simultaneously with the test determinations. This curve was used to determine the amount of reducing sugar in the test solution before and after hydrolysis. In this way the amount of cellulose (presumed to be the predominant hydrolysable polysaccharide in these preparations) was determined.

Assay of radioactivity. After assay, each lipid from experiments with labelled precursor was diluted with known carrier amounts of appropriate unlabelled material by using synthetic plastoquinone-9, authentic castaprenol mixture and stigmasterol respectively. The labelled fractions were chromatographed on suitable thin-layer chromatographic systems to constant specific activity. Though the stigmasterol-enriched fractions almost certainly contained other sterols, previous work (Wellburn, 1966) has shown both plastoquinone-9 and castaprenols to be pure after this treatment. After a final assay of the lipid materials, the specific activities were recorrected to the original amounts of labelled material. All counting was carried out with a Packard Tri-Carb liquid-scintillation counting system (model 314EX; Packard Instrument Co., La Grange, Ill., U.S.A.) The scintillation solution consisted of 0.5% (w/v) 2,5-diphenyloxazole and 0.3% (w/v) 1,4-bis-2-(4-methyl-5-diphenyloxazol-2-yl)benzene in toluene. All counts of radioactivity were corrected for background, quenching and the efficiency of counting.

Thin-layer chromatography. Thin layers (275 μ) of silica gel G (E. Merck A.-G., Darmstadt, Germany) were used in all cases. A 50% (v/v) solution of benzene in light petroleum (b.p. 40–60°) was used as developing solvent for the purification of plastoquinone-9 (R_F 0.58). Castaprenols had R_F 0.64 when chloroform was used as solvent, and sterol was purified by using 3% (v/v) methanol in benzene as solvent (R_F 0.43). These compounds were located by viewing under ultraviolet light the chromatoplate after spraying with a 0.01% (w/v) solution of fluorescein in ethanol (Dunphy, Whittle & Pennock, 1965).

RESULTS AND DISCUSSION

During studies of intracellular distribution, the preparation of a good specimen of a particular component is generally to the detriment of the other subfractions. Numerous studies have described

subfractions of very indefinite constitution, often in terms of their centrifugal characteristics. In the present series of experiments, an attempt was made to prepare two good specimens of subcellular components, namely intact chloroplasts by one of the best recognized techniques available and cell walls by one of the few successful methods described.

The recovery of castaprenols and plastoquinone from the cell-wall preparation, in each case, has been corrected for contamination derived from the chloroplasts. In making this correction it has been assumed that chlorophyll occurs only in the chloroplasts and that the concentrations of castaprenols and plastoquinone/mg. of chlorophyll are the same in intact or ruptured chloroplasts. The attainment of a direct relationship between whole-leaf lipids and those in the cell-wall preparations has been enabled by the expression of all lipid concentrations in terms of 'cellulose' or hydrolysable reducing sugar. Plastoquinone, determined in molar terms, is probably a measure of plastoquinone-9 together with plastoquinone-4, which has been reported in horse-chestnut leaves (Eck & Trebst, 1963). The castaprenols, however, cannot be represented in molar terms because of their heterogeneity. The difference between the whole-leaf lipid and the combined lipids from the chloroplasts and the cell walls is enumerated under the heading 'residue', which would include such subcellular components as mitochondria, ribosomes and nuclei.

The yields of plastoquinone and castaprenols from the various fractions are shown in Table 1. The results for plastoquinone at 16 and 20 weeks support the conclusion of others, that most of the plastoquinone of the plant cell is associated with the chloroplasts (Bishop, 1958; Crane, 1959). However, at 24 weeks over one-third of the plastoquinone was not associated with the chloroplasts.

The bulk of the castaprenols also accompanies plastoquinone within the chloroplast envelope at 16

Table 1. *Change in the distribution of plastoquinone and castaprenols in subcellular fractions from Aesculus leaves with age*

Values in parentheses are the quantities expressed as percentages of the total recovered from the subcellular fractions. Age is given in weeks after terminal-bud unfolding.

	Age (weeks)	Quantities		
		Chloroplasts	Cell walls	Residue
Plastoquinone (μ moles/g. of tissue cellulose)*	16	7.70 (82.5)	0.11 (1.2)	1.52 (16.3)
	20	6.41 (87.3)	0.33 (4.5)	0.60 (8.2)
	24	4.64 (58.4)	2.10 (27.7)	1.40 (13.9)
Castaprenols (mg./g. of tissue cellulose)	16	2.67 (86.7)	0.29 (9.4)	0.12 (3.9)
	20	3.08 (80.6)	0.60 (15.7)	0.14 (3.7)
	24	2.64 (50.3)	2.33 (44.4)	0.28 (5.3)

* 1 μ mole of plastoquinone-9 $\equiv$ 0.748 mg.

Table 2. *Distribution of radioactivity in plastoquinone and castaprenols of subcellular fractions obtained from Aesculus leaves having metabolized [2-¹⁴C]mevalonate 20 weeks and 24 weeks after terminal-bud unfolding*

	Age of leaves (weeks)	10 ⁻³ × Specific activities		
		Chloroplasts	Cell walls	Residue
Plastoquinone (disintegrations/min./μmole)	20	0.057	0.022	0.255
	24	0.012	0.344	1.452
Castaprenols (disintegrations/min./mg.)	20	0.077	0.273	14.49
	24	0.033	0.958	4.04

Table 3. *Effect of ultrasonic treatment on the metabolism of [2-¹⁴C]mevalonate by isolated chloroplasts from Aesculus 18 weeks after terminal-bud unfolding*

Constituent		Total amount (μmoles or mg.)		10 ⁻³ × Sp. activity (disintegrations/min. per mg. or per μmole)	
		2A	2B	2A	2B
Treated with ultrasound	Expt. no. ...				
	Plastoquinone (μmoles)	0.216	0.068	2.55	7.45
	Castaprenols (mg.)	33.3	23.3	8.07	10.80
	Free sterols (mg.)	23.0	16.0	7.40	6.90
Not treated with ultrasound	Chlorophyll (μmole)	0.820	0.565		
	Plastoquinone (μmoles)	0.193	0.088	0.88	1.60
	Castaprenols (mg.)	38.0	20.0	3.18	3.03
	Free sterols (mg.)	22.0	12.0	6.32	5.09
	Chlorophyll (μmole)	0.798	0.611		

or 20 weeks, but most of the remaining castaprenols appears to be localized within the cell walls, unlike the remaining plastoquinone in the 'residue'. At 24 weeks almost half of the castaprenol was associated with the cell walls. The non-chlorophyll-associated plastoquinone and castaprenol of the 'residue' are unlikely to be derived from disruption of mature chloroplasts, but may be associated with underdeveloped proplastids to be found in the extrachloroplastidic portion of the plant cell.

Whether the changes in subcellular distribution with age are involved in the onset of senescence, or as a result of it, is not yet clear. The increase of non-chlorophyll-associated plastoquinone towards the end of the season may indicate the disruption of the cellular localization of those systems responsible for the maintenance of photosynthetic activity.

Analyses for sterols were carried out only in Expt. 2. The sterol concentrations of the chloroplasts (Table 3) were surprisingly high when compared with the results obtained by Mercer & Treharne (1966) with chloroplasts of a different species.

The amount of radioactivity associated with plastoquinone and the castaprenols in the two later stages is also shown in Table 2. The low incorporation of radioactivity into these compounds within

the chloroplasts is presumably due to the relative impermeability of the chloroplast membrane to mevalonate, as shown by Goodwin & Mercer (1963). Apart from this, on examination of the specific activities of both isoprenoids it seems likely that a biosynthetic site for each also exists outside the chloroplast. These sites are probably associated with the 'soluble' fraction of the 'residue'. The results are consistent with a portion of the compounds, particularly the castaprenols, being formed in the 'soluble' fraction followed by transfer to the cell walls.

Table 3 (Expts. 2A and 2B) shows the effect of ultrasonic treatment on the incorporation of activity from [2-¹⁴C]mevalonate into plastoquinone and the castaprenols by isolated horse-chestnut chloroplasts. Those results for plastoquinone demonstrate the relative inability of mevalonate to cross intact chloroplast envelopes and partake in the biosynthesis of a chloroplastidic terpenoid. Sterols, however, shown to be biosynthesized outside the chloroplast (Mercer & Goodwin, 1962, 1963; Treharne, Mercer & Goodwin, 1964) show little alteration with respect to incorporation of activity after rupture of the chloroplast envelope. Assuming the membrane of the unruptured chloroplasts to be relatively impermeable to mevalonic acid, it is clear

that the chloroplast preparation contains sterol-synthesizing enzymes of extraplastidic origin. Similarly, some contamination of the chloroplast preparation by the extraplastidic castaprenol-synthesizing system is indicated. Nevertheless, the threefold increase in incorporation of radioactivity into the castaprenols, in suspensions of ruptured chloroplasts over non-ruptured chloroplasts, would indicate a site of polyisoprenoid alcohol biosynthesis within the chloroplast as well as outside.

Chloroplast disruption may be a natural phenomenon in aging leaves and involve a concomitant redistribution of the various enzymes involved in chloroplast terpenoid formation. This may explain the presence in older leaves of apparent sites of biosynthesis outside the chloroplast envelope of two isoprenoid constituents found and formed predominantly inside the chloroplast in younger leaves.

The rate of biosynthesis of polyisoprenoid alcohols is invariably slow. Indeed, the use of mature leaf tissue in this study is enforced as only then does biosynthesis occur to any significant extent. Polyisoprenoid alcohols accumulate in older plant tissue and have been shown to be specifically associated with the chloroplasts of the cell. It is therefore relevant that, as leaves age, the rise in the concentration of the castaprenols, both inside and outside the chloroplast, at 20 and 24 weeks is accompanied by a fall in chlorophyll and plastoquinone concentrations (Wellburn & Hemming, 1966*a,b*). It may well be that at this stage there is a gradual loss of control of metabolism of the common precursor geranylgeranyl pyrophosphate that leads to an increase in the activity of the castaprenol-synthesizing system and a decrease in activities of the phytol- and plastoquinone-synthesizing systems. It is also possible that the increased castaprenol-synthesizing activity outside the chloroplast is associated with 'natural' rupture of some of the chloroplasts. Both processes could be significant factors in the senescence of leaves.

A.R.W. was in receipt of a Medical Research Council Research studentship.

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