Biosynthesis of the Prenyl Side Chains of Plastoquinone and Related Compounds in Maize and Barley Shoots

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The incorporation of ^{14}C by etiolated maize and barley shoots exposed to light of $^{14}\text{CO}_2$ and $[2\text{-}^{14}\text{C}]$ mevalonic acid into phyloquinone, plastoquinone, ubiquinone, α -tocopherolquinone and α -tocopherol was examined. In maize (the principal tissue studied) it was demonstrated that ^{14}C from $[2\text{-}^{14}\text{C}]$ mevalonic acid is incorporated into phyloquinone, plastoquinone and ubiquinone. α -Tocopherol and α -tocopherolquinone, although undoubtedly labelled from this substrate, were not purified completely. As expected, ^{14}C from $^{14}\text{CO}_2$ was incorporated into all components examined. Ozonolytic degradation studies showed that ^{14}C from $[2\text{-}^{14}\text{C}]$ mevalonic acid was incorporated specifically into the prenyl side chains of plastoquinone and ubiquinone, and from this it was inferred that mevalonic acid can be regarded as the specific distal precursor to the prenyl portions of all terpenoid quinones occurring in plant tissues. From a comparison of the relative incorporation of ^{14}C from $^{14}\text{CO}_2$ and $[2\text{-}^{14}\text{C}]$ mevalonic acid into the intra- and extra-chloroplastidic terpenoids evidence was obtained consistent with the tenet that the prenyl portions of the chloroplastidic quinones phyloquinone and plastoquinone, along with β -carotene, are biosynthesized within the confines of the chloroplast, the side chain of the extraplastidic ubiquinone and phytosterols being synthesized elsewhere within the cell. The results obtained for the incorporation of ^{14}C from $^{14}\text{CO}_2$ and $[2\text{-}^{14}\text{C}]$ mevalonic acid into α -tocopherol and α -tocopherolquinone were not readily interpretable with regard to the site of synthesis of these compounds.

Griffiths, Threlfall & Goodwin (1967) have reported on the nature of the terpenoid quinones and chromanols occurring in young maize and barley shoots. Maize shoot, the principal tissue studied, was shown to contain phyloquinone, plastoquinone, α -tocopherolquinone and ubiquinone-9 (i.e. ubiquinone with 9 isoprene units/mol.). The presence of α -tocopherol and γ -tocopherol (Green, 1958) was also confirmed. It was demonstrated that the concentration of the chloroplastidic quinones phyloquinone, plastoquinone and α -tocopherolquinone was related directly to the degree of chloroplast development. Ubiquinone-9, as expected, was relatively unaffected by the degree of development of this organelle. Rather surprisingly, no relationship could be shown between the concentration of α -tocopherol (which is believed to be localized in the chloroplast) and chloroplast development. Barley shoots, although not studied as extensively, gave essentially similar results.

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The investigations we report now are concerned with the biosynthesis of the prenyl side chains of the phytoquinones in maize and barley shoots. The aspects considered are (a) the nature of the specific distal precursor and (b) the sites of synthesis of the isoprenoid side chains.

MVA† is recognized as the specific distal precursor of the basic C_5 unit from which all terpenoids are derived. It is incorporated into the prenyl side chain of ubiquinone in rat tissues (Gloor, Schindler & Wiss, 1960) and yeasts and moulds (Raman, Sharma, Jayaram & Ramasarma, 1965). In maize shoots it is a precursor of, among other terpenoids, β -carotene (Goodwin, 1958), 3β -hydroxy sterols (Mercer & Goodwin, 1963) and the phytol side chain of chlorophyll (Mercer & Goodwin, 1962). Thus our first aim was to determine whether MVA is incorporated into the nonaprenyl side chains of plastoquinone and ubiquinone-9 and the partly saturated tetraprenyl side chain of phyloquinone by maize and barley shoots. The incorporation of this substrate into the saturated tetraprenyl-containing

† Abbreviations: MVA, mevalonic acid; E/P, solution of diethyl ether in light petroleum (b.p. 40–60°).

compounds α -tocopherolquinone and α -tocopherol was also examined.

Goodwin (1958), while studying the incorporation of $[2-^{14}\text{C}]\text{MVA}$ into the chloroplast pigment β -carotene by maize seedlings, under conditions where a massive synthesis of this pigment takes place [on illumination of etiolated (dark-grown) seedlings, cf. phyloquinone, plastoquinone and α -tocopherolquinone (Griffiths *et al.* 1967)], was unable to demonstrate that this substrate was an effective precursor of the pigment, i.e. incorporation of label was less than was expected in view of the synthesis taking place. On the other hand, radioactivity was rapidly incorporated into the 3β -hydroxy sterols. When $^{14}\text{CO}_2$ was tried, ^{14}C was readily incorporated into the β -carotene, but only poorly into the sterols. This result was more compatible with the large synthesis of pigment taking place. Similar results were obtained for the phytol side chain of chlorophyll (Mercer & Goodwin, 1962). These experiments led Goodwin & Mercer (1963) to propose that within the photosynthetic plant cell there are two sites of terpenoid biosynthesis. One site is in the chloroplast, giving rise to β -carotene, the phytol side chain of chlorophyll and any other terpenoids normally associated with this organelle, e.g. phyloquinone, plastoquinone and α -tocopherolquinone; the second site is outside the chloroplast and gives rise to the phytosterols and, in our case, the nonaprenyl side chain of ubiquinone-9. It is suggested that the physical separation of these two sites is brought about by the chloroplast membrane's being impermeable or only partly permeable to MVA and subsequent members of the terpenoid biosynthetic sequence. Thus $[2-^{14}\text{C}]\text{MVA}$, on entering the plant cell, can readily join the extraplastidic biosynthetic sequence leading to sterol formation, but cannot effectively penetrate the chloroplast membrane and join the biosynthetic sequences leading to β -carotene and the phytol side chain of chlorophyll. Conversely, ^{14}C from $^{14}\text{CO}_2$, after fixation by the chloroplast, is readily incorporated into β -carotene and phytol.

It follows from these proposals that labelling of the terpenoid portions of the chloroplastidic quinones phyloquinone, plastoquinone and α -tocopherolquinone (and α -tocopherol) after exposure of the plants to $^{14}\text{CO}_2$ and $[2-^{14}\text{C}]\text{MVA}$ should be similar to that of β -carotene, whereas labelling of ubiquinone-9 would be expected to be the same as that of the phytosterols. This problem formed the second part of our investigations.

The experimental system used for all experiments reported in the present paper was greening excised etiolated (dark-grown) maize or barley shoots. The seedlings were germinated in the dark for 6–7 days, excised at the node, and exposed to the appropriate substrate with continuous illumination for up to

36 hr. (24 hr. is in fact the optimum period). The characteristics of this system are: (i) a large incremental synthesis of chlorophyll, β -carotene and the chloroplastidic quinones, plastoquinone, α -tocopherolquinone and phyloquinone; (ii) no significant net synthesis of the phytosterols (unesterified 3β -hydroxy sterols), α -tocopherol and ubiquinone.

The results of these experiments have been reported briefly by Griffiths, Threlfall & Goodwin (1964) and Threlfall, Griffiths & Goodwin (1964).

EXPERIMENTAL

Radiochemicals

$[2-^{14}\text{C}]\text{MVA}$ and $\text{Ba}^{14}\text{CO}_3$ were purchased from The Radiochemical Centre, Amersham, Bucks.

Biological methods

Biological material. *Zea mays* (var. South African White Horse Tooth) seeds were purchased from Gunsons (Seeds) Ltd., London, E. 1. *Hordeum sativum* Cb763 (var. Proctor) seeds were a gift from the Welsh Plant Breeding Station, Aberystwyth.

Cultivation of seedlings. Etiolated 6- or 7-day-old seedlings were used for all experiments. They were grown as described by Griffiths *et al.* (1967).

Exposure of etiolated seedlings to ^{14}C -labelled substrates. Goodwin (1958) demonstrated that $[2-^{14}\text{C}]\text{MVA}$, one of the precursors studied, cannot penetrate the root system of maize, but can enter the excised shoots. In view of this excised shoots were used for all the experiments described.

The shoots after excision and presentation of the substrate were exposed to continuous illumination for the period of the experiments. The effect of illumination on the quinone, carotenoid and sterol content of etiolated shoots has been reported elsewhere (see the introduction and Griffiths *et al.* 1967). The numbers of shoots taken (usually 100 for maize and 1000 for barley) and the radioactivity administered are given in the text.

(1) $[2-^{14}\text{C}]\text{MVA}$. Etiolated shoots were excised at the node and collected into bundles of 100 shoots held together by an elastic band. The excised ends were then immersed in a shallow glass beaker containing $[2-^{14}\text{C}]\text{MVA}$ in water (see the Results section for activities and volumes of water used).

The shoots were then exposed to continuous illumination for the experimental period as described by Griffiths *et al.* (1967). To encourage transpiration from the leaf surface, and thus facilitate uptake of the substrate, the shoots were kept in a continuous air stream, created by a small electric fan placed a few feet away. Within 3 hr. of illumination the formation of chlorophyll in the shoots was apparent to the naked eye.

(2) $^{14}\text{CO}_2$. $^{14}\text{CO}_2$ -incorporation experiments were carried out in a large desiccator (internal diameter at flange 20 or 29 cm.), the radioactive gas being generated by the addition of lactic acid to $\text{Ba}^{14}\text{CO}_3$.

Tap water was placed in the desiccator to a depth of 1 in., and this was covered with two layers of wire mesh, which served to support the excised shoots. Provision was made

for introducing lactic acid through the neck of the desiccator into a small weighing bottle containing $\text{Ba}^{14}\text{CO}_3$ and a few millilitres of water.

$^{14}\text{CO}_2$ was produced by the dropwise addition of lactic acid to the suspension of $\text{Ba}^{14}\text{CO}_3$. At the end of the experimental period a continuous stream of N_2 was passed through the apparatus to remove unfixed $^{14}\text{CO}_2$.

Illumination was provided by means of a Philips (250 w) 'cold light' placed 1 ft. above the lid of the desiccator. The use of this light prevented overheating of the air within the desiccator and also reduced fogging of the glass surfaces due to condensation of water vapour. Excised shoots under these conditions 'greened up' similarly to, if not better than, plants in a normal atmosphere exposed to tungsten lamps.

Analytical methods

Extraction of lipid. At the end of the experimental period the shoots were rinsed thoroughly with distilled water and dried between layers of filter paper. The lipid was then extracted by our routine method (Griffiths *et al.* 1967). In Expt. III, where incorporation of substrate into different regions of the shoot was investigated, the shoots, after being damp-dried, were cut into three portions representing tip, mid-shoot and base. These portions were then extracted by the usual method.

After the ethereal extract had been dried over Na_2SO_4 , the solution was made up to known volume and samples were taken for chlorophyll assay, saponification or ^{14}C assay as required. The extract was evaporated to dryness under N_2 in a tared flask. The extract (referred to below as total lipid extract) was then subjected to chromatography on a column of acid-washed alumina (see below).

Saponification. Determining the total incorporation of ^{14}C by counting a sample of the total lipid extract, although expedient, is not always reliable, since (a) the extraction procedure is such that the washing steps may not remove all unutilized $[2\text{-}^{14}\text{C}]\text{MVA}$ and (b) the coloured extracts can cause appreciable quenching on liquid-scintillation counting. In Expt. II, where a reliable value for the incorporation of $[2\text{-}^{14}\text{C}]\text{MVA}$ was required, samples of the total lipid extracts were saponified; this in effect means that the values obtained refer only to the incorporation of ^{14}C into the unsaponifiable lipids. However, since all the compounds under investigation belong to this class of lipids, this seemed an effective means of measuring incorporation. [Although saponification leads to destruction of the terpenoid quinones and chromanols present in the extract there is no reason to doubt that the side chain fragment(s) will be extracted by this procedure. Again, in $[2\text{-}^{14}\text{C}]\text{MVA}$ experiments, the incorporation of ^{14}C into quinones and chromanols is quantitatively relatively insignificant.]

The saponification was carried out by dissolving the sample, contained in a 25 ml. conical flask, in the minimum volume of ethanol (not less than 10 ml.). Aq. 60% (w/v) KOH was added (0.1 ml./10 ml. of ethanol) and the mixture boiled for 20 min. At the end of this time the mixture was diluted with 3–4 vol. of water and then extracted three times with ether (1 vol. each time). The combined ethereal extracts were washed free of alkali with distilled water, dried over anhydrous Na_2SO_4 and evaporated to dryness under N_2 in a tared flask. Samples were then taken for ^{14}C assay.

Isolation of quinones and related terpenoids. (1) Preliminary column chromatography. The resolution of the

total lipid extracts into quinone-containing fractions was carried out by column chromatography on acid-washed Brockmann grade III alumina (anionotropic; Woelm). The columns were developed successively with 0.25%, 1%, 3%, 5%, 8%, 12% and 20% E/P. The conditions of development and the elution sequence of the various quinones and related compounds have been reported previously (Griffiths *et al.* 1967; see also Table 1). All the fractions, before being evaporated to dryness, were made up to a suitable volume and samples taken for ^{14}C assay. The amounts of any components under investigation present in the fraction were also estimated, i.e. β -carotene (0.25%), plastoquinone (1%), ubiquinone (5%), α -tocopherolquinone (20%), sterol esters (1%) and 3β -hydroxy sterols (12%). Phylloquinone and α -tocopherol cannot be estimated at this stage (see below).

(2) Purification of plastoquinone, ubiquinone, phylloquinone, α -tocopherol and α -tocopherolquinone. The quinones and chromanols in the various fractions were purified by combinations of column, adsorptive thin-layer and reversed-phase thin-layer chromatography. Kieselgel G (E. Merck A.-G., Darmstadt, Germany) was the adsorbent used for all thin-layer systems. In Expts. III and IV plates impregnated with Rhodamine 6G were used. This obviated the need to use reagent sprays and makes for a more precise location of these compounds (Griffiths *et al.* 1967). The preparation and development of thin-layer plates, and the location and recovery of the various compounds, have been reported previously (Threlfall & Goodwin, 1967; Griffiths *et al.* 1967).

(a) Plastoquinone. This quinone and the esterified sterols were eluted by 1% E/P in the preliminary chromatography. Samples of this fraction were taken to determine the total concentration of these two components. Total plastoquinone was estimated by reduction of a sample in ethanol with NaBH_4 (Threlfall & Goodwin, 1967). Total esterified sterol was determined by saponification of a sample and preparation of the digitonide of the resulting non-saponifiable material, after which the sterol content of the digitonide was estimated (see below).

The fraction was rechromatographed on a column (of the same dimensions as the column on which the preliminary chromatography was carried out) of acid-washed Brockmann grade II alumina, developed with 1% and 2% E/P. Plastoquinone and esterified sterol were eluted by 2% E/P. Although this chromatography made little difference to the specific activity of the quinone expressed as total counts in the fraction it did remove a yellow pigment (adsorbed strongly on top of the column), making the subsequent purification steps easier. The plastoquinone-containing fraction recovered from the column was quantitatively chromatographed on thin layers developed with light petroleum (b.p. 40–60°)–benzene (3:2, v/v). Plastoquinone (R_F 0.3) was normally visible on the developed plate as a faint yellow band. In some cases, however, the quinone had to be detected with leucomethylene blue, sprayed on the marker spots (Threlfall & Goodwin, 1967). The sterol ester fraction was eluted as the zone between plastoquinone and the solvent front and examined by the procedure outlined below.

The plastoquinone at this stage was virtually radiochemically pure (see the Results section for a full discussion as to the validity of this assumption), and, after ^{14}C assay and spectroscopic determination of the amount of quinone present, the specific activity was calculated. As a final

purification step the plastoquinone preparations were chromatographed quantitatively on reversed-phase thin layers [paraffin-impregnated plates with aq. 95% (v/v) acetone as developing solvent]. The recovered 'plastoquinone-paraffin' mixture was separated on acid-washed Brockmann grade III alumina (paraffin and plastoquinone eluted by light petroleum and 1% E/P respectively) and the specific activity of the recovered quinone determined. In all cases this value was of the same order as that of the starting material.

In some experiments the purified quinone was reduced to the quinol and chromatographed on reversed-phase thin layers with aq. 95% (v/v) acetone as solvent (plastoquinol has R_F 0.8). The quinol was located by spraying the plate with Emmerie-Engel reagent (Threlfall & Goodwin, 1967). Neatran strips were then prepared of the appropriate area (see below) and either scanned for ^{14}C radioactivity or used to prepare radioautographs.

The quinol was prepared by dissolving the sample in a few millilitres of ethanol and adding Zn dust and a few drops of conc. HCl. After standing for 1 min. the mixture was filtered, diluted with water and the quinol extracted with light petroleum (b.p. 40–60°). Invariably during this procedure slight reoxidation of the quinol took place, so that two ^{14}C -labelled spots were obtained on thin-layer chromatography, corresponding to the quinone and quinol. A more convenient method, leading to a quantitative conversion of quinone into quinol, is to dissolve the sample in a small volume of ethanol and add a small crystal of NaBH_4 before spotting (Dilley, 1964).

(b) Ubiquinone. Ubiquinone isolation and purification proved to be difficult since this compound occurred in a heavy fraction (5% E/P) and constituted only about 1–2% of the weight. The bulk of the fraction is made up of a long-chain saturated alcohol (Griffiths *et al.* 1967). An additional difficulty to radiochemical work, especially when using [^{14}C]MVA, is the occurrence in this fraction of phytosterol precursors, i.e. members of the 4,4-dimethyl and 4 α -methyl steroid groups. During the investigation two methods were used for the purification of this quinone.

In Expts. I (pilot experiment) and II an attempt was made to decrease the weight of the fraction by a procedure involving acetylation. The fraction was taken up in a minimum volume of cyclohexane (2 ml.) and acetylated at room temperature overnight by the addition of 2 drops of pyridine and 2 ml. of acetic anhydride. The following day, the reaction mixture was poured into ice-cold 5*N*-HCl (10 ml.) and extracted with ether. The extract was washed three times with 5*N*-HCl and finally with water until free from Cl^- ions. After removal of the solvent the fraction was chromatographed on acid-washed Brockmann grade III alumina (Woelm), developed with 1%, 3% and 5% E/P. Ubiquinone was present in the 5%-E/P fraction.

The ubiquinone sample was then dissolved in a minimum volume of hot cyclohexane and quantitatively chromatographed on thin layers developed with benzene-chloroform (1:1, v/v) (R_F 0.5). With the ubiquinone samples that still contained some alcohol, the cyclohexane solution was allowed to stand before spotting, when the alcohol precipitated out as a white solid. The ubiquinone-containing supernatant was then chromatographed. The recovered sample was assayed for ^{14}C radioactivity and quinone content and then rechromatographed on reversed-phase thin layers with aq. 95% (v/v) acetone as developing solvent (ubi-

quinone has R_F 0.45). The ubiquinone was separated from the contaminating paraffin by chromatography on a column of acid-washed Brockmann grade III alumina (Woelm). Paraffin was eluted by 1% E/P and ubiquinone by 5% E/P.

In Expt. III, where Rhodamine 6G-impregnated plates (see above) were available and a more precise location of the quinone band could be carried out, the fraction was dissolved in hot cyclohexane, allowed to stand and the supernatant spotted. The recovered ubiquinone was then run on the thin-layer systems described above. Rhodamine 6G-impregnated plates cannot be used for reversed-phase systems since the solvents used cause the dye to migrate. In these cases non-impregnated plates are used for development and then sprayed with a 0.2% (w/v) solution of Rhodamine 6G in acetone. After being dried, the plates are examined under u.v. light as before.

(c) Phylloquinone. Phylloquinone was eluted along with β -carotene by 0.25% E/P. It is not possible at this stage to assay the vitamin by its spectral characteristics. The fractions were rechromatographed on columns of acid-washed Brockmann grade II alumina (Griffiths *et al.* 1967). β -Carotene was eluted by 1% E/P and phylloquinone by 2% E/P. The phylloquinone-containing fraction was assayed for naphthaquinone content and ^{14}C radioactivity. The quinone was then purified by repeated thin-layer chromatography on Rhodamine 6G-impregnated plates with benzene-light petroleum (b.p. 40–60°) (2:3, v/v) as developing solvent (Griffiths *et al.* 1967).

(d) α -Tocopherol. This chromanol was found in the 3%-E/P fraction after preliminary chromatography. The fraction was first chromatographed on Rhodamine 6G-impregnated plates with benzene-chloroform (1:1, v/v) as developing solvent (R_F 0.47). The recovered chromanol was estimated spectroscopically (Threlfall & Goodwin, 1967) and assayed for ^{14}C radioactivity. The chromanol was then oxidized to the *p*-benzoquinone, α -tocopherolquinone, by the method of Dilley & Crane (1963) and chromatographed on a column of acid-washed Brockmann grade III alumina (Woelm). α -Tocopherolquinone was eluted by 20% E/P. The quinone was finally chromatographed quantitatively on thin layers developed with ethyl acetate-benzene (3:22, v/v; R_F 0.48). The quinone was estimated spectroscopically by reduction with NaBH_4 (Threlfall & Goodwin, 1967), and the ^{14}C assayed at each step.

(e) α -Tocopherolquinone. This quinone is eluted by 20% E/P during the preliminary alumina chromatography. It was purified by chromatography on Rhodamine 6G-impregnated plates developed with ethyl acetate-benzene (3:22, v/v).

(3) Purification of β -carotene and 3 β -hydroxy sterols (free and esterified). (a) β -Carotene. This pigment was eluted, along with squalene and phylloquinone, by 0.25% E/P. The pigment was resolved from phylloquinone, but not squalene, by rechromatography on columns of acid-washed Brockmann grade II alumina (Woelm) (see the purification of phylloquinone). The β -carotene fraction was then chromatographed on thin layers developed with benzene-light petroleum (b.p. 40–60°) (1:19, v/v) (R_F 0.6). The amount of β -carotene was estimated spectrophotometrically (Threlfall & Goodwin, 1967) and assayed for ^{14}C radioactivity. The samples for ^{14}C assay were transferred to counting vials and bleached under u.v. light before counting. In Expt. II sufficient purified β -carotene was obtained to carry out a crystallization under the same conditions as used

by Goodwin (1958). The crystals and mother liquors had the same specific activities, which demonstrated that the above procedure gives a radiochemically pure sample.

(b) β -Hydroxy sterols (unesterified). β -Hydroxy sterols were purified via their digitonides. Stigmasterol, β -sitosterol and campesterol (in maize) are eluted by 12% E/P during the preliminary chromatography of lipid extracts. The fraction was split into two samples: A (for 100 maize shoots usually one-tenth was taken, i.e. about 2mg. of sterol) and B (the remainder), for determination of the total sterol in the original fraction and the specific activity of the sterol respectively. The β -hydroxy sterols in both samples were separated from other lipid material by precipitation as their digitonides, prepared by the method of Windaus (1909). The digitonides were then washed twice with each of the following solvent mixtures: acetone-ether (1:1, v/v), ether-ethanol (1:1, v/v) and ether. This procedure removes any carotenoids and other contaminating lipids that may be present.

The sterol content of sample A was now determined by the Liebermann-Burchard colour reaction as described by Davies (1963). The washed purified sample of free sterol as sterol digitonide, contained in a 10ml. centrifuge tube, was dissolved in 3ml. of acetic acid and 6ml. of ice-cold reagent [acetic anhydride-conc. H_2SO_4 (19:1, v/v)] added. The reaction mixture was incubated in darkness for 33 min. at 25° to allow development of the blue colour. The colour developed was determined spectrophotometrically at 620 μ and the sterol concentration read off a standard graph.

The free sterol was regenerated from sample B by using pyridine to break the complex (Williams & Goodwin, 1966). The recovered sterol was then dissolved in chloroform and samples were taken for ^{14}C assay and estimation of sterol present by the Liebermann-Burchard reaction. Adsorptive thin-layer chromatography, in a system [ethyl acetate-benzene (3:22, v/v)] that will separate sterols into three groups, the 4,4-dimethyl sterols, the 4 α -methyl sterols and the phytosterols with no 4-methyl groups (Goad & Goodwin, 1966), followed by X-ray radioautography, showed the presence of only one area of fogging, corresponding to the position of β -hydroxy sterols.

(c) Esterified β -hydroxy sterols. Esterified sterols are eluted along with plastoquinone by 1% E/P. The total β -hydroxy sterols in this fraction were determined by taking a sample (usually one-tenth) of the fraction, saponifying and preparing the digitonide of the unsaponifiable material. The amount of sterols was then estimated by the Liebermann-Burchard reaction (see above; the volumes used were reduced threefold). The specific activity of the sterol was determined by taking the material recovered after thin-layer chromatography of the fraction to isolate plastoquinone (see above), saponifying, preparing the digitonide of the unsaponifiable material and, after regeneration of the free sterols, estimation of the specific activity (see above). The samples were saponified by dissolving in 2ml. of ethanol and 0.2ml. of aq. 60% (w/v) KOH and heating on a water bath (100°) for 20 min. The mixture was then diluted with water (3-4 vol.) and extracted three times with ether (1 vol.). The ethereal extracts were combined and washed free of alkali with water, dried over anhydrous Na_2SO_4 and evaporated to dryness under N_2 .

The sterol esters of maize have, as their major component members of the β -hydroxy sterol class, stigmasterol and β -sitosterol (Griffiths, 1965); however, they also contain

small amounts of highly radioactive 4,4-dimethyl and 4 α -methyl sterols (D. R. Threlfall, unpublished work). Thus, though the digitonide washing procedure would remove any 4,4-dimethyl sterols (Ohta & Shimizu, 1957), 4 α -methyl sterols are not removed (L. J. Goad, personal communication), so that the final specific-activity determination represents β -hydroxy sterols plus 4 α -methyl sterols.

Ozonolytic degradation of plastoquinone and ubiquinone. The ^{14}C radioactivity in the isoprenoid side chains of plastoquinone and ubiquinone was determined by an ozonolytic degradation method. In outline (see Scheme 1) this involves reductive acetylation of the quinone, followed by ozonolysis of the diacetate and mild reductive cleavage of the ozonide. The laevulic aldehyde derived from the isoprenoid side chain is isolated and assayed as its bis-DNP-hydrazine derivative.

The sample (3-10mg.) consisting of ^{14}C -labelled quinone plus carrier quinone was, after determination of its specific activity, reductively acetylated (Binkley, McKee, Thayer & Doisy, 1940).

The resulting diacetate ('di-*O*-acetyldihydroquinone') was dissolved in 2-3ml. of ethyl chloride, cooled to -70°, and ozone bubbled through the solution until a persistent blue colour appeared. The mixture was left for 30 min. at -70°, and then excess of ozone was removed by bubbling N_2 -free O_2 through the solution until the blue colour had been discharged.

The ozonide was cleaved by adding 100mg. of Zn dust followed by 2 drops of acetic acid and allowing the mixture to stand for 1-2 hr. The mixture was then filtered into a ice-cold receiver and the Zn dust washed with 2ml. of water followed by a few millilitres of ethyl chloride. The laevulic-aldehyde was isolated from the filtrate, converted into its bis-DNP-hydrazine derivative and purified by the procedures described by Morton *et al.* (1958).

The purity of the derivative was ascertained by: (i) chromatography on thin layers of Kieselgel G developed with ethyl acetate-benzene (3:7, v/v) (Burgos, Hemming, Pennock & Morton, 1963); (ii) determination of m.p. (232-234°, uncorr.); (iii) $E_{1\text{cm}}^{1\%}$ (911 at 357 μ in chloroform).

Finally, about 1mg. of the crystals was taken up in 10ml. of chloroform. The amount of derivative present was estimated spectroscopically by taking a 1ml. sample and, after diluting it tenfold, measuring the extinction at 357 μ (Burgos *et al.* 1963). The ^{14}C radioactivity of the sample was determined by taking 1ml. samples of the original solution and evaporating them to dryness in a counting vial. The residue was then redissolved in 1-2ml. of dioxan, made up to 20ml. with scintillator fluid and counted. The concentration of derivatives for ^{14}C assay is critical and should not exceed 100 $\mu\text{g.}/20\text{ml.}$ of scintillator fluid, otherwise quenching will occur.

A satisfactory yield of the bis-DNP-hydrazine derivative of laevulic aldehyde can be obtained by this procedure starting with only 0.5mg. of quinone. The ^{14}C assay procedure, i.e. scintillation counting, is limited, however, by the ability to assay the ^{14}C radioactivity of the bis-DNP-hydrazine derivative of laevulic aldehyde in view of the quenching shown by such derivatives in anything but dilute solutions. However, derivatives with low specific activities can be assayed in the proportional counter.

Spectrophotometry. Ultraviolet and visible spectra were

In most radiochemical investigations involving terpenoid quinones and related compounds, i.e. carotenoids and sterols, the preferred method for the isolation of pure compounds is saponification, since this removes many contaminating lipids and makes for an easier purification procedure. However, experiments had shown that saponification of plant material, in particular maize and barley shoots, even in the presence of pyrogallol (as antioxidant), led to extensive destruction of some of the compounds concerned in our studies (Griffiths, 1965). This meant that in the investigations described below the terpenoid component had to be isolated from total lipid extracts. The problems associated with the isolation and purification of quinones from total lipid extracts of maize

Table 1. *Incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{mevalonic acid}$ and $^{14}\text{CO}_2$ into lipids of maize and barley shoots*

Expt. I. Batches of etiolated maize (2×250) and barley (2×1250) shoots were excised and exposed to either $[2\text{-}^{14}\text{C}]\text{MVA}$ ($30\text{ }\mu\text{C}$ in 100 ml. of water) or $^{14}\text{CO}_2$ (see below for radioactivities given) for 36 hr. with continuous illumination. At the end of this period the total lipid was extracted and chromatographed on a column of Brockmann grade III acid-washed alumina. The concentrations of the quinone components at the end of the experiment in the shoots exposed to $[2\text{-}^{14}\text{C}]\text{MVA}$ were $910\text{ }\mu\text{g.}$ of plastoquinone, $298\text{ }\mu\text{g.}$ of ubiquinone-9 and $424\text{ }\mu\text{g.}$ of α -tocopherolquinone for maize and $1\text{-}546\text{ mg.}$ of plastoquinone for barley. The values for the shoots exposed to $^{14}\text{CO}_2$ were similar.

Fraction	Maize shoots (5-day-old)*						Barley shoots				Terpenoid constituents
	$[2\text{-}^{14}\text{C}]\text{MVA}$ ($30\mu\text{C}$)			$^{14}\text{CO}_2$ ($330\mu\text{C}$)			$[2\text{-}^{14}\text{C}]\text{MVA}$ ($30\mu\text{C}$)		$^{14}\text{CO}_2$ ($1000\mu\text{C}$)		
	Wt. (mg.)	$10^{-3} \times ^{14}\text{C}$ activity (counts/min.)	Wt. (mg.)	$10^{-3} \times ^{14}\text{C}$ activity (counts/min.)	Wt. (mg.)	$10^{-3} \times ^{14}\text{C}$ activity (counts/min.)	Wt. (mg.)	$10^{-3} \times ^{14}\text{C}$ activity (counts/min.)	Wt. (mg.)	$10^{-3} \times ^{14}\text{C}$ activity (counts/min.)	
Total lipid extract	260	8720	434	3950	349	19100	598	6740			
Column fractions											
Light petroleum	3.8	130	46.3	65	22.4	192	114	118		β -Carotene, phyloquinone and squalene	
1% E/P	3.7	1450	9.2	131	14.3	4730	6.5	431		Plastoquinone and sterol esters	
3% E/P	2.8	154	2.5	35	23.5	682	5.3	106		α -Tocopherol	
5% E/P	16.2	1147	26.4	189	56.3	4110	55.8	895		Ubiquinone-9, sterol precursors (4,4-dimethyl and 4 α -methyl sterols) and γ -tocopherol in the maize	
8% E/P	1.4	669	9.7	68	17.4	1990	89.3	372		β -Hydroxy sterols (stigmasterol, β -sitosterol and campesterol)	
12% E/P	21.5	2062	32.8	82	18.1	2944	29.2	211		α -Tocopherolquinone	
20% E/P	6.2	153	6.5	24	2.4	563	10.4	164			
Recovered (%)	22	66	31	15	44	80	51	35			

* In a repeat of the maize $[2\text{-}^{14}\text{C}]\text{MVA}$ experiment 87 6-day-old etiolated shoots were exposed to $30\text{ }\mu\text{C}$ of $[2\text{-}^{14}\text{C}]\text{MVA}$ in 100 ml. of water for 36 hr. with continuous illumination. The amount of plastoquinone at the end of the period was $773\text{ }\mu\text{g.}$

and barley shoots have already been extensively reported (Griffiths *et al.* 1967).

A pilot experiment was carried out in which incorporation of ^{14}C from $^{14}\text{CO}_2$ and $[2\text{-}^{14}\text{C}]\text{MVA}$ into the various lipid fractions of maize and barley shoots was investigated. The substrates were administered for 36 hr. to 'greening' excised shoots; the rationale behind the choice of this experimental system has already been explained (see the introduction). Table 1 summarizes the pertinent details of the incorporation of radioactivity into the total lipid extracts and various chromatographic fractions. Included in Table 1, for the sake of clarifying later discussion, is a list of the various terpenoids found in each fraction. In the maize experiment 5-day-old shoots were used instead of the normal 6-7-day-old shoots, and so the amounts of the various components are lower than in other experiments reported.

Table 1 shows that of the ^{14}C administered the incorporation into the total lipid from $[2\text{-}^{14}\text{C}]\text{MVA}$ was, after correcting for the fact that only the D-isomer is utilized (Popják, 1959), 26% in maize and 57% in barley. On the other hand, ^{14}C from $^{14}\text{CO}_2$ was only poorly incorporated: 0.54% and 0.30% for the maize and barley respectively. This difference reflects the diverse metabolic pathways entered by carbon dioxide, i.e. incorporation into proteins, lipids and carbohydrates, whereas MVA is specifically incorporated into terpenoids, almost all of which are present in the lipid fraction.

On chromatography the recovery of ^{14}C was

Fig. 1. Rechromatography on alumina columns of plastoquinone-containing fractions obtained from maize shoots exposed to (a) $^{14}\text{CO}_2$ or (b) $[2\text{-}^{14}\text{C}]\text{MVA}$. The 1%-E/P fractions obtained from the preliminary chromatography of lipid extracts on alumina were rechromatographed on columns of Brockmann grade II alumina. Plastoquinone-containing fractions (2% E/P) were collected as eight 25 ml. subfractions and each fraction was assayed for plastoquinone content ($\circ$) and ^{14}C radioactivity (Δ) (values uncorrected for machine efficiency).

greater from $[2\text{-}^{14}\text{C}]\text{MVA}$ than $^{14}\text{CO}_2$, again reflecting specific incorporation of ^{14}C from the former into terpenoids. The fractions from maize contained most of the radioactivity from $[2\text{-}^{14}\text{C}]\text{MVA}$ in the 1%-E/P, 5%-E/P and 12%-E/P fractions, indicating that this compound is readily incorporated into sterol esters (see below), early sterol precursors and 3β -hydroxy sterols respectively. Barley lipid failed to show this clear-cut distribution, considerable activity occurring in the 8%-E/P fraction. Incorporation of ^{14}C from $^{14}\text{CO}_2$, as would be expected, tended to be more general.

Isolation of radiochemically pure plastoquinone. Since the problems met with in the isolation of $[^{14}\text{C}]\text{plastoquinone}$ were similar for both plant tissues, only the maize results are presented below.

With $[2\text{-}^{14}\text{C}]\text{MVA}$ 4.35% of the radioactivity was recovered in the plastoquinone-containing fractions, whereas with $^{14}\text{CO}_2$ only 0.018% of the radioactivity was incorporated into this fraction. The initial stages in the purification of plastoquinone were carried out by rechromatography of the fractions on columns of Brockmann grade II alumina as described in the Experimental section and by Griffiths *et al.* (1967). Plastoquinone was eluted by 2% E/P, the fraction being collected as eight 25 ml. subfractions; the fractions were analysed for quinone content and ^{14}C radioactivity. The results obtained are presented in Fig. 1. The graphs show clearly that the ^{14}C radioactivity in the fractions from the plants exposed to $[2\text{-}^{14}\text{C}]\text{MVA}$ did not coincide with the quinone content of the various fractions, the bulk of the radioactivity being in a material that runs before and with plastoquinone. The results for the $^{14}\text{CO}_2$ experiment showed that the ^{14}C radioactivity of the fractions coincided exactly with the quinone elution pattern.

To achieve further purification, the plastoquinone subfractions containing the highest amounts of quinone were chromatographed as short bands (3 cm.) on thin layers (Kieselgel G) with benzene-light petroleum (b.p. 40-60°) (2:3, v/v), and Neatan strips were prepared of the developed chromatoplates (see the Experimental section). The strips were then scanned for ^{14}C radioactivity and finally sprayed with a solution of antimony pentachloride. On spraying, two bands became apparent on the strip. One (violet-brown, R_f 0.55), corresponding to plastoquinone showed up immediately, whereas a second band (brown, R_f 0.9) appeared only after heating at 90° for 1-2 min. When the strips were aligned with the corresponding radioactive scans (Fig. 2), it was found that the major peak of radioactivity on the strip from the $[2\text{-}^{14}\text{C}]\text{MVA}$ experiment coincided with the compound having R_f 0.9, whereas on the strip from the $^{14}\text{CO}_2$ experiment the bulk of the radioactivity was associated with the plastoquinone zone.

Fig. 2. Radioscans of Neatan strips prepared from thin-layer chromatograms of plastoquinone-containing samples, purified by column chromatography, from the $^{14}\text{CO}_2$ (a) and $[2\text{-}^{14}\text{C}]\text{MVA}$ experiments. The scanning conditions used for the $[2\text{-}^{14}\text{C}]\text{MVA}$ experiment were: count rate, 3000 counts/min.; collimator width, $\frac{1}{8}$ in.; time constant, 20 sec.; scanning speed, 0.75 in./hr. The conditions for the $^{14}\text{CO}_2$ experiment were: count rate, 300 counts/min.; collimator width, $\frac{1}{8}$ in.; time constant, 40 sec.; scanning speed, 0.75 in./hr. After scanning the strips were sprayed with SbCl_5 (see the text). O, Origin; PQ, plastoquinone; SE, sterol esters; SF, solvent front.

The remaining plastoquinone subfractions from the two experiments were combined into their respective groups and chromatographed quantitatively on thin layers as above, and the plastoquinone was recovered. At the same time the area corresponding to the highly radioactive zone in the strip from the $[2\text{-}^{14}\text{C}]\text{MVA}$ experiment was recovered from samples from both the $^{14}\text{CO}_2$ and $[2\text{-}^{14}\text{C}]\text{MVA}$ experiments, combined and kept for investigation. Unfortunately, during these procedures an excessive loss of plastoquinone from the $[2\text{-}^{14}\text{C}]\text{MVA}$ experiment occurred such that only 40 $\mu\text{g.}$ was recovered compared with 290 $\mu\text{g.}$ from the $^{14}\text{CO}_2$ experiment. To obtain more plastoquinone a second incubation with MVA was carried out, the outline of which is given in Table 1.

Table 2 shows that the recovered plastoquinone from the $^{14}\text{CO}_2$ experiment exhibited only a small decrease in specific activity when compared with the specific activity of the original fraction expressed in terms of plastoquinone; the plastoquinone from the $[2\text{-}^{14}\text{C}]\text{MVA}$ experiment, on the other hand, showed a large decrease in specific activity owing to removal of the highly radioactive material. The recovered plastoquinone was mixed

with carrier and recrystallized several times from ethanol at room temperature, the specific activity of the crystals and mother liquors being determined after each crystallization. Table 2 shows that during this procedure no appreciable change in the specific activities of the plastoquinone from the $^{14}\text{CO}_2$ or the $[2\text{-}^{14}\text{C}]\text{MVA}$ experiment took place; in view of this it was concluded that the samples were radiochemically pure. Additional confirmation of this was obtained by subjecting the samples to reversed-phase thin-layer chromatography both as the quinone and after reduction to the corresponding quinol; on preparing Neatan strips and either scanning for radioactivity or preparing X-ray plates, which are more sensitive for the detection of small amounts of ^{14}C radioactivity, only one peak or area of fogging, corresponding exactly to the position on the strip of the quinone or quinol, was observed in all cases.

In the light of these results it was concluded that in future work the plastoquinone samples could in fact be regarded as radiochemically pure after quantitative adsorptive and reversed-phase thin-layer chromatography (see the Experimental section).

Investigation into the nature of the radioactive plastoquinone contaminant. The material recovered from the $^{14}\text{CO}_2$ and the $[2\text{-}^{14}\text{C}]\text{MVA}$ experiments was combined to give a reasonable amount of material to work with. From the outset, in view of the colour produced by spraying Neatan strips containing this material with antimony pentachloride, the substance was suspected of being a sterolic-type compound (or compounds), and from its elution pattern during chromatography probably an esterified sterol. This view was strengthened by the fact that esterified ergosterol had been detected in a corresponding fraction obtained during the chromatography of lipid extracts from *Euglena* (Threlfall & Goodwin, 1967).

The combined sample (about 5 mg.) was saponified and the liberated sterols were purified via their digitonides.

Samples of this material, when treated with the Liebermann-Burchard colour reagent, gave a bluish colour indicative of a slow-reacting sterol. Further, on thin-layer chromatography of this sample against authentic 3β -hydroxy sterol markers (β -sitosterol and stigmasterol) and exposure of the developed plate to iodine vapour, the material was found to have an R_f value identical with that of the markers. Gas-liquid chromatography of the material against a range of authentic sterol samples showed the presence of compounds having the same retention times as β -sitosterol and stigmasterol, with the latter predominating. Work subsequently carried out by ourselves and other members of the Department now suggests that, although β -sitosterol and stigmasterol are quantitatively the

Table 2. *Purification of plastoquinone samples*

Expt. I. Plastoquinone (PQ) from the appropriate fractions (1% E/P) obtained on chromatography of the total lipid extracts from maize and barley shoots that had been exposed to $^{14}\text{CO}_2$ or $[2\text{-}^{14}\text{C}]\text{MVA}$ were purified by column and thin-layer chromatography followed by crystallization.

Purification step*	Maize shoots				Barley shoots			
	$[2\text{-}^{14}\text{C}]\text{MVA}\dagger$		$^{14}\text{CO}_2$		$[2\text{-}^{14}\text{C}]\text{MVA}$		$^{14}\text{CO}_2$	
	Sp. activity (counts/min./mg.)		Sp. activity (counts/min./mg.)		Sp. activity (counts/min./mg.)		Sp. activity (counts/min./mg.)	
	2020 000 (0.77)		142 000 (0.93)		3 060 000 (1.55)		251 000 (1.72)	
Preliminary column chromatography†	103 000		107 000		43 000 (1.18)		111 000 (1.52)	
Thin-layer chromatography†	110 $\mu\text{g.}$ of $[^{14}\text{C}]\text{PQ}$ + 5.21 mg. of PQ		255 $\mu\text{g.}$ of $[^{14}\text{C}]\text{PQ}$ + 4.50 mg. of PQ		705 $\mu\text{g.}$ of $[^{14}\text{C}]\text{PQ}$ + 5.02 mg. of PQ		486 $\mu\text{g.}$ of $[^{14}\text{C}]\text{PQ}$ + 5.02 mg. of PQ	
Crystallization	Crystals	Liquors	Crystals	Liquors	Crystals	Liquors	Crystals	Liquors
First	2061	1971	5930	6170	4190	6040	9750	12100
Second	2109	2197	5840	5980	4865	4940	10020	10140
Third	—	—	—	—	4770	4740	9340	10260

* The second purification step, i.e. column chromatography on Brockmann grade II alumina, has been omitted.

† The plastoquinone sample used was from the repeat incubation (see Table 1 and the text).

‡ Values in parentheses are the amounts (mg.) of plastoquinone recovered at each step.

predominant sterols, small amounts of highly active 4,4-dimethyl and 4 α -methyl sterols are also present in the original fraction. These may be partially precipitated by the digitonide purification technique and would contribute radioactivity to the so-called purified 3 β -hydroxy sterol ester fractions of experiments described below.

Examination of the ubiquinone-containing fractions. The difficulties associated with isolating a radiochemically pure sample of ubiquinone have already been discussed (see the Experimental section).

Purification of the unesterified 3 β -hydroxy sterols. Examination of the 12%-E/P fractions (see the Experimental section) showed that the 3 β -hydroxy sterols accounted for all the weight and most of the ^{14}C radioactivity in the original fraction. Gas-liquid chromatography showed that the sterols present were stigmasterol and β -sitosterol, although it now appears that small amounts of campesterol and cholesterol are also present (Hamman, 1966; L. J. Goad, personal communication).

A summary of the results from these experiments are given in Table 3. If at this stage the assumption is made that $[2\text{-}^{14}\text{C}]\text{MVA}$ is incorporated into the solanesol side chain of plastoquinone, and that $^{14}\text{CO}_2$ is incorporated into the entire molecule, then the incorporation of the ^{14}C -labelled substrates into plastoquinone and the 3 β -hydroxy sterols obtained in these experiments (Table 3) is quantitatively as predicted by the proposals of Goodwin & Mercer

(1963) to explain the regulation of terpenoid synthesis in higher plants.

Thus in maize 6.4% of the radioactivity of the added D- $[2\text{-}^{14}\text{C}]\text{MVA}$ was recovered in the 3 β -hydroxy sterols whereas only 0.2% was found in the isolated plastoquinone, even though a rapid synthesis of plastoquinone occurred during the experimental period. In barley the corresponding values were 12.1% and 0.2% respectively. In contrast, in the maize $^{14}\text{CO}_2$ experiment only 0.009% of the radioactivity appeared in the 3 β -hydroxy sterols whereas 0.014% was recovered in the plastoquinone. Similarly, in the barley $^{14}\text{CO}_2$ experiment, a reversal of the $[2\text{-}^{14}\text{C}]\text{MVA}$ pattern was seen; only 0.005% of the radioactivity was recovered in the 3 β -hydroxy sterols whereas 0.009% was found in the plastoquinone.

Biosynthesis of plastoquinone and ubiquinone side chains in relationship to other terpenoids

The preferential incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{MVA}$ into sterols and from $^{14}\text{CO}_2$ into plastoquinone, observed in the previous experiment, had already been predicted by the 'compartmentalization theory' of Goodwin & Mercer (1963). However, this theory is only strictly applicable to the terpenoid side chain of the plastoquinone molecule. Thus it was necessary to show that $^{14}\text{CO}_2$ is effectively used for the synthesis of the whole molecule, whereas $[2\text{-}^{14}\text{C}]\text{MVA}$ is only used for the specific synthesis of the isoprenoid side chain.

Table 3. Incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{mevalonic acid}$ and $^{14}\text{CO}_2$ into plastoquinone and $3\beta\text{-hydroxy sterols}$ of maize and barley shoots
 Expt. I. Etiolated maize and barley shoots were excised and exposed to $[2\text{-}^{14}\text{C}]\text{MVA}$ or $^{14}\text{CO}_2$ with continuous illumination for 36 hr. (Tables 1 and 2).

Fraction	Maize shoots						Barley shoots					
	$[2\text{-}^{14}\text{C}]\text{MVA}$ ($20\mu\text{C}$)*			$^{14}\text{CO}_2$ ($300\mu\text{C}$)			$[2\text{-}^{14}\text{C}]\text{MVA}$ ($30\mu\text{C}$)			$^{14}\text{CO}_2$ ($1000\mu\text{C}$)		
	Incorporation†		$10^{-3} \times \text{Sp. activity (counts/min./mg.)}$	Incorporation†		$10^{-3} \times \text{Sp. activity (counts/min./mg.)}$	Incorporation†		$10^{-3} \times \text{Sp. activity (counts/min./mg.)}$	Incorporation†		$10^{-3} \times \text{Sp. activity (counts/min./mg.)}$
	(μC)	(%)		(μC)	(%)		(μC)	(%)		(μC)	(%)	
Total lipid extract	3.36	22.4	—	1.78	0.54	—	8.6	57.4	—	3.04	0.304	—
Plastoquinone	0.036	0.2	103	0.045	0.014	107	0.03	0.2	43	0.086	0.009	111
$3\beta\text{-Hydroxy sterols}^\ddagger$	0.96	6.4	137	0.03	0.009	2.06	1.82	12.1	218	0.052	0.005	6.3

* The maize $[2\text{-}^{14}\text{C}]\text{MVA}$ values are from the repeat incubation (see Table 1 and the text).

† Expressed as a percentage of the D-isomer administered.

‡ The weights of the purified $3\beta\text{-hydroxy sterols}$ were: maize, 15.6 mg. ($[2\text{-}^{14}\text{C}]\text{MVA}$) and 27.8 mg. ($^{14}\text{CO}_2$); barley, 18.6 mg. ($[2\text{-}^{14}\text{C}]\text{MVA}$) and 18.2 mg. ($^{14}\text{CO}_2$).

Again, to provide a sound basis for comparison, the incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{MVA}$ and $^{14}\text{CO}_2$ into plastoquinone was studied in relation to the incorporation into other terpenoids of chloroplastidic ($\beta\text{-carotene}$) and extraplastidic (ubiquinone-9 and sterols) origin. The uptake of $[2\text{-}^{14}\text{C}]\text{MVA}$ was also examined from the point of view of time, since it was felt that this could provide valuable information on the early steps of terpenoid biosynthesis in greening tissues. The tissue chosen for this and all subsequent investigations was maize. The 6-day-old etiolated maize shoots were excised and exposed with continuous illumination either to $[2\text{-}^{14}\text{C}]\text{MVA}$ for various times (3, 6 and 12 hr.) up to 24 hr. or to $^{14}\text{CO}_2$ for 24 hr.; 24 hr. was chosen since previous experiments (Griffiths *et al.* 1967) had shown that this is the optimum period for chloroplastidic terpenoid synthesis. The tissues were then examined for the incorporation of radioactivity into the various components. Table 4 and Fig. 3 show the effect of light on the synthesis of chlorophyll, $\beta\text{-carotene}$, plastoquinone and sterols, and also the incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{MVA}$ into the unsaponifiable lipid. In agreement with previous experiments it was found that light stimulated the formation of chlorophyll, $\beta\text{-carotene}$ and plastoquinone, but had little effect on the amounts of total sterol (esterified and unesterified).

Incorporation of ^{14}C from $^{14}\text{CO}_2$ and $[2\text{-}^{14}\text{C}]\text{mevalonic acid}$ into plastoquinone. The results (Fig. 3 and Table 4) show that even after 3 hr., before appreciable net synthesis of plastoquinone has taken place, radioactivity is being effectively incorporated into this quinone. Further, a plot of the total radioactivity incorporated into plastoquinone against the amount of plastoquinone present at any given time was a straight line (Fig. 4), demonstrating that the substrate was available to the plastoquinone-synthesizing sites at the same rate throughout the experiment. Extrapolation of the curve should give the weight of the original non-radioactive endogenous plastoquinone, assuming no appreciable turnover, which in view of the massive synthesis occurring is reasonable. This value, $210\mu\text{g.}$, is lower than the value at zero time ($254\mu\text{g.}$), suggesting that appreciable photodestruction or enzymic destruction (or both) of the quinone occurs during the initial period of illumination. Such a decrease has in fact been measured in other experiments (Griffiths *et al.* 1967).

Again $^{14}\text{CO}_2$ was found to be more effectively utilized for plastoquinone synthesis, when compared with the phytosterols (Table 4), than $[2\text{-}^{14}\text{C}]\text{MVA}$.

Incorporation of ^{14}C from $^{14}\text{CO}_2$ and $[2\text{-}^{14}\text{C}]\text{mevalonic acid}$ into ubiquinone. Ubiquinone was purified by the procedures described above. Table 5 summarizes the results.

Table 4. Incorporation of ^{14}C from $[2-^{14}\text{C}]$ mevalonic acid (with time) and $^{14}\text{CO}_2$ into unsaponifiable lipid, plastoquinone and phytosterols (unesterified and esterified)Expt. II. Etiolated 6-day-old maize shoots (975) were excised and exposed with continuous illumination either to $[2-^{14}\text{C}]$ MVA for various times up to 24 hr. or to $^{14}\text{CO}_2$ for 24 hr.

Results all expressed in terms of 100 shoots										
Substrate	Time of exposure (hr.)	No. of shoots analysed	Plastoquinone			Phytosterols				
			Unesterifiable lipid	10 ⁻³ × Sp.		Unesterified*		Esterified		
				10 ⁻³ × ¹⁴ C activity (counts/min.)	Amount (μg.)	activity (counts/min.)	Amount (mg.)	10 ⁻³ × Sp. activity (counts/min.-mg.)	Amount (mg.)	10 ⁻³ × Sp. activity (counts/min.-mg.)
[2- ¹⁴ C]MVA (6 μC/20 ml. of water/100 shoots)	0	100	0	254	0	15.2	0	1.21	0	
	3	100	954	253	9.3	2.4	15.1	11.4	91	
	6	100	1680	283	12.2	3.5	15.9	44.1	163	
	12	100	2010	400	24.8	9.9	14.7	63.9	265	
	24	300	5575	785	40.7	32.0	22.9	96	394	
	24	275	3000	860	179.6	154.3	25.1	3.9	18.3	
¹⁴ CO ₂ (550 μC)										

* 3β -Hydroxy sterols. Fig. 3. Effect of light on concentrations of chlorophyll, plastoquinone, β -carotene, unesterified 3β -hydroxy sterols and incorporation into unsaponifiable material of ^{14}C radioactivity from $[2-^{14}\text{C}]$ MVA by excised etiolated maize shoots. O, Chlorophyll; Δ , plastoquinone; $\square$, unesterified 3β -hydroxy sterols; $\bullet$, β -carotene; $\blacktriangle$, ^{14}C radioactivity. Fig. 4. Incorporation of ^{14}C radioactivity from $[2-^{14}\text{C}]$ MVA into plastoquinone.

Degradation of plastoquinone and ubiquinone. Recoveries of $[^{14}\text{C}]$ plastoquinone after purification on the 24 hr. samples from the $^{14}\text{CO}_2$ and the $[2-^{14}\text{C}]$ MVA experiments were 1.058 mg. and 1.016 mg. respectively. Approx. 10 mg. of carrier plastoquinone was added to each sample and after one crystallization they were degraded and the radioactivity in the side chains was determined (Table 6).

The ^{14}C radioactivity in the side chains of the plastoquinone molecules was calculated as follows. Scheme 1 shows the expected labelling pattern for the intact molecule and degradation products of plastoquinone labelled from $[2-^{14}\text{C}]$ MVA. From

Table 5. *Purification of ubiquinone*

Expt. II. Ubiquinone was isolated from the appropriate fractions (5% E/P) obtained on chromatographing the total lipid extracts from maize shoots that had been exposed to $[2-^{14}\text{C}]\text{MVA}$ or to $^{14}\text{CO}_2$ for 24 hr. with continuous illumination (Table 4).

Purification step	$[2-^{14}\text{C}]\text{MVA}$			$^{14}\text{CO}_2$		
	Wt. (mg.)	Ubiquinone ($\mu\text{g.}$)	$10^{-3} \times \text{Sp. activity}^*$ (counts/min./mg.)	Wt. (mg.)	Ubiquinone ($\mu\text{g.}$)	$10^{-3} \times \text{Sp. activity}^*$ (counts/min./mg.)
Column chromatography	44.4	—	—	34.7	—	—
Acetylation and column chromatography	10.9	426	1584	9.3	503	199
Precipitation from petroleum	5.2	356	1013	6.0	474	94
Thin-layer chromatography						
(I) Benzene-chloroform (1:1, v/v)		316	460	—	381	23.1
(II) Reversed-phase partition		157	495	—	187	24.8

* Specific activities calculated in terms of ubiquinone content.

this it is seen that the laevulic aldehyde arises from the degradation of seven biogenetic isoprene units plus two fragments of biogenetic isoprene units from each end of the side chain, i.e. it is equivalent to eight biogenetic isoprene units. If we assume that the radioactivity throughout the side chain is uniformly repetitive [in fact we have a mean value for eight units, and this only means assuming that the radioactivity in the labelled carbon from the terminal isoprene unit (liberated as acetone in the degradation procedure) does not differ markedly from the other eight], then the total radioactivity in the side chain will be given by : $9 \times \text{counts/min.}/\mu\text{mole}$ of laevulic aldehyde. Similarly, for plastoquinone labelled from $^{14}\text{CO}_2$, if the assumption is made that the radioactivity in the two carbon atoms adjacent to the ring and the three carbons from the terminal isoprene unit does not differ from that in the other isoprene units, then the total radioactivity in the side chains will be given by the same expression.

For plastoquinone from the $[2-^{14}\text{C}]\text{MVA}$ experiment, the radioactivity in the side chain should equal the original radioactivity (counts/min./ μmole) of the plastoquinone molecule, and this is found to be the case (Table 6). However, in the $^{14}\text{CO}_2$ experiment, the radioactivity should be incorporated uniformly throughout the molecule, the ^{14}C radioactivity in the side chain accounting for 82% of the total radioactivity. The result obtained in this case (77%) is only slightly lower than expected, although in similar experiments with maize shoots values of 82% have been obtained (D. R. Threlfall, unpublished work).

Ubiquinone was degraded by the same procedure.

In this case only the sample from the $[2-^{14}\text{C}]\text{MVA}$ experiment was examined, since the distribution of radioactivity in the ubiquinone from the $^{14}\text{CO}_2$ experiment was of little consequence in interpreting the overall experiment. In agreement with the results obtained for plastoquinone ^{14}C from $[2-^{14}\text{C}]\text{MVA}$ was found to be incorporated only into the ubiquinone side chain (Table 6).

Incorporation of ^{14}C from $^{14}\text{CO}_2$ and $[2-^{14}\text{C}]\text{mevalonic acid}$ into β -carotene and 3β -hydroxy sterols. These terpenoids were isolated as reference compounds since their behaviour towards the two substrates was known (Goodwin, 1958; Treharne, Mercer & Goodwin, 1966). For β -carotene only the samples from 24 hr. exposure to substrate were examined. The purification procedure has already been outlined above. Table 9 summarizes the results.

The 3β -hydroxy sterols, which in maize occur in both the free and esterified form, the former predominating, were examined for radioactivity by the methods already outlined above. The concentration of sterol and incorporation of radioactivity were followed throughout the $[2-^{14}\text{C}]\text{MVA}$ incubation; for the $^{14}\text{CO}_2$ experiment only a 24 hr. sample was examined.

Table 4 summarizes the results obtained. Although the sterols were purified via their digitonides, a method that is supposed to yield only 3β -hydroxy sterols, the sterols from the ester fraction almost certainly contained in addition 4α -methyl derivatives. 4,4-Dimethyl sterols, which are also present in this fraction, would be removed during the digitonide washing procedure (Ohta & Shimizu, 1957). The unesterified sterol consists entirely of β -sitosterol, stigmasterol and a trace of campesterol,

Table 6. *Ozonolytic degradation of plastoquinone and ubiquinone*

Expt. II. Purified plastoquinone and ubiquinone isolated from maize shoots that had been exposed to [2-¹⁴C]MVA or to ¹⁴CO₂ for 24 hr. were mixed with the appropriate carrier material and subjected to a scheme of ozonolytic degradation (Scheme 1).

Sample	Wt. (mg.)	Sp. activity (counts/min./mg.)	Carrier added (mg.)	Sp. activity after crystallization (counts/min./μmole)	Laevulinic aldehyde derivative*		Activity in side chain (%)
					(counts/min./μmole)†	(× 9 or 9.94) (counts/min./μmole)‡	
Plastoquinone ([2- ¹⁴ C]MVA expt.)	1.016	40700	9.73	2795	304	2736	98
Plastoquinone (¹⁴ CO ₂ expt.)	1.058	179600	9.54	13246	1137	10233	77
Ubiquinone ([2- ¹⁴ C]MVA expt.)	0.146	495000	4.29	13970	1379	13700	98

* Bis-DNP-hydrazine derivative.

† Mean of three determinations.

‡ In error ubiquinone-9 instead of ubiquinone-10

sample. The factor 9.94 allows for this.

having been resolved completely from 4,4-dimethyl and 4 α -methyl sterols by preliminary column chromatography (see Table 1). The results show that during the experiment the amounts of esterified 3 β -hydroxy sterols showed a slight increase with time of exposure to light, the free 3 β -hydroxy sterols being relatively unaffected.

From the results given in Table 6 it would appear that some synthesis of 3 β -hydroxy sterols occurs during the last 12 hr. of illumination. This result is due entirely to the conscious selection of the slightly better shoots, i.e. broader rather than longer, for the 24 hr. samples. In a more carefully controlled experiment it was shown that under these conditions no net synthesis of 3 β -hydroxy sterols takes place. The values obtained after the incubation of shoots for 0.5, 1, 3, 6 and 24 hr. in the light were 23.4, 23.6, 23.4, 22.5 and 22.5 mg./100 shoots respectively. Throughout the experimental period the specific activity of the sterol esters was always significantly higher (owing probably to the presence of 4 α -methyl sterols) than the 3 β -hydroxy sterols (Table 4).

During the early part of the incubation the total radioactivity in the sterols esters was similar to that in the free sterols, but as time progressed the amount in the free sterols relative to the sterol esters rose. The results suggested that perhaps the 3 β -hydroxy sterol esters were precursors of the free 3 β -hydroxy sterols. The type of role envisaged was one of transport of the newly synthesized sterols to the specific location in the cell at which they are required, e.g. under our conditions the developing chloroplast.

Radioactivity from ¹⁴CO₂ was poorly incorporated into both the free and esterified 3 β -hydroxy sterols, the esterified form having the higher specific activity.

The pertinent results for the complete experiment are given in Table 9.

Distribution of ¹⁴C radioactivity within the shoot

Although experiments with whole shoots have advantages with regard to the amount of material available for analysis they also have certain disadvantages, one of which is that 'greening up' is not uniform over the whole shoot and thus results obtained from a gross analysis may not be a true reflection of the situation in the photosynthetic regions.

To clarify the situation, incorporation of ¹⁴C from [2-¹⁴C]MVA and ¹⁴CO₂ into various intra- and extra-plastidic terpenoids was examined in relation to the different regions of chloroplast development, as measured by the amount of chlorophyll, within the shoot (Table 7).

Table 7. Incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{mevalonic acid}$ and $^{14}\text{CO}_2$ into plastoquinone, ubiquinone and $3\beta\text{-hydroxy sterols}$ isolated from different regions of the maize shoot

Expt. III. Etiolated maize shoots (2×100) were excised and exposed with continuous illumination to either $[2\text{-}^{14}\text{C}]\text{MVA}$ ($10\mu\text{C}$ in 20ml. of water) or to $^{14}\text{CO}_2$ ($200\mu\text{C}$) for 24 hr. The shoots were then dissected into three regions, i.e. base, mid and tip, and analysed.

Component	$[2\text{-}^{14}\text{C}]\text{MVA}$ ($10\mu\text{C}$)			$^{14}\text{CO}_2$ ($200\mu\text{C}$)		
	Base (5cm.)	Mid (7.5cm.)	Tip (6.3cm.)	Base (5cm.)	Mid (7.5cm.)	Tip (6.3cm.)
Lipid extract (mg.)	85	112	54	87	103	77
Unsaponifiable lipid (mg.)	17	47	33	Not determined		
$10^{-3} \times ^{14}\text{C}$ activity (counts/min.)	1726	818	270	Not determined		
Chlorophyll (mg.)	2.1	10.4	4.2	2.6	10.7	5.3
$\beta\text{-Carotene}$ ($\mu\text{g.}$)	52	216	114	23	149	176
Plastoquinone ($\mu\text{g.}$)	99	519	396	52	420	330
$10^{-3} \times \text{Sp. activity of plastoquinone}$ (counts/min./mg.)	41.5	33.9	35.7	396	442	488
$10^{-3} \times \text{Sp. activity of ubiquinone}$ (counts/min./mg.)	Mean:	36.7		Mean:	458	
		427.4		Not determined		
$3\beta\text{-Hydroxy sterols}$ (mg.)	7.7	6.1	4.0	7.6	8.5	6.0
$10^{-3} \times \text{Sp. activity of } 3\beta\text{-hydroxy sterols}$ (counts/min./mg.)	65.2	49.9	55.9	3.45	3.84	3.29
	Mean:	57.8		Mean:	3.55	

Incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{mevalonic acid}$ and $^{14}\text{CO}_2$ into phytol-containing components

The investigations so far described have been concerned with the quinones containing nonaprenyl side chains. It was decided to determine whether the terpenoid side chains of phylloquinone, $\alpha\text{-tocopherolquinone}$ and $\alpha\text{-tocopherol}$, which can be regarded as arising from phytol, are, in accordance with their intracellular localization, biosynthesized within the chloroplast. From the results of Griffiths *et al.* (1967) it would be expected that phylloquinone and $\alpha\text{-tocopherolquinone}$, both of which show an incremental synthesis in the experimental system used, would behave in the $^{14}\text{CO}_2$ and $[2\text{-}^{14}\text{C}]\text{MVA}$ experiments in a similar manner to plastoquinone. $\alpha\text{-Tocopherol}$ is somewhat difficult to tie in since under our conditions no appreciable synthesis takes place; however, since it seems reasonable *a priori* to expect this compound to be in a state of equilibrium with $\alpha\text{-tocopherolquinone}$, the incorporation of radioactivity into $\alpha\text{-tocopherol}$ would be expected to mimic that into $\alpha\text{-tocopherolquinone}$.

Table 8 outlines the experiment and gives the results obtained for the various stages of the purification procedures. It is seen that phylloquinone can be regarded as radiochemically pure after the first thin-layer chromatography; the purity was checked by running a sample on reversed-phase thin layers (Threlfall & Goodwin, 1967) and preparing a radioautograph, when only one area of fogging corresponding exactly to the position of phylloquinone was observed. $\alpha\text{-Tocopherolquinone}$ and $\alpha\text{-tocopherol}$ were not purified

to constant specific activity, but merely to a stage where it became apparent that they failed to fit into either the sterol or plastoquinone type of incorporation pattern.

The pertinent results are given in Table 10. Again, to provide a basis for comparison, the incorporation of ^{14}C radioactivity into $\beta\text{-carotene}$ and sterols was determined.

DISCUSSION

MVA has been shown to be an effective precursor of the ubiquinone side chain in rats (Gloor *et al.* 1960) and in yeasts and moulds (Raman *et al.* 1965). We have shown that in maize and barley shoots this compound is incorporated into phylloquinone (only reported here for maize), plastoquinone and ubiquinone-9. Degradation studies showed that in maize $[2\text{-}^{14}\text{C}]\text{MVA}$ was specifically incorporated into the nonaprenyl side chain of plastoquinone and ubiquinone-9 (Table 6). Phylloquinone was not degraded, but it would seem reasonable to expect that the ^{14}C radioactivity is incorporated solely into the partly saturated tetraprenyl side chain of this naphthaquinone.

The biosynthetic routes leading to the formation of terpenoid quinones are not as yet clear, but the consensus of opinion would seem to support the view that in the quinones with unsaturated side chains the prenyl unit, as the pyrophosphate, is synthesized independently of the nucleus by sequential addition of isopentenyl pyrophosphate units to the isoprenoid-chain primer dimethylallyl pyrophosphate, to form, in plastoquinone and ubiquinone-9 synthesis, solanesyl pyrophosphate, or in ubiquinone-10 synthesis, decaprenyl pyrophosphate.

Table 8. *Isolation and purification of phylloquinone, α -tocopherol and α -tocopherolquinone from maize shoots exposed to [2- 14 C]mevalonic acid or to $^{14}\text{CO}_2$*

Expt. IV. Etiolated maize shoots (2×100) were excised and exposed with continuous illumination either to [2- 14 C]MVA ($6 \mu\text{C}$ in 20 ml. of water) or to $^{14}\text{CO}_2$ ($263 \mu\text{C}$) for 24 hr. The phylloquinone, α -tocopherol and α -tocopherolquinone were isolated from the appropriate fractions (0.25, 3 and 20% E/P respectively) obtained on chromatographing the lipid extracts on columns of Brockmann grade III acid-washed alumina.

Sample	Phylloquinone			α -Tocopherol			α -Tocopherolquinone		
	Purification step*	Wt. ($\mu\text{g.}$)	$10^{-3} \times \text{Sp. activity (counts/min./mg.)}$	Purification step*	Wt. ($\mu\text{g.}$)	$10^{-3} \times \text{Sp. activity (counts/min./mg.)}$	Purification step*	Wt. ($\mu\text{g.}$)	$10^{-3} \times \text{Sp. activity (counts/min./mg.)}$
[2- 14 C]MVA ($6 \mu\text{C}$)	Grade II alumina column	112	31.8	Thin layer (II) (chromanol)	226	136	Thin layer (III)	162	12.3
	Thin layer (I)	87	28.1	Grade III alumina column	114	52.7	Thin layer (II)	92	3.8
	Thin layer (I)	62	26.4	Thin layer (III) (quinone)	67	26.5			
	Grade II alumina column	92	44.3	Thin layer (II) (chromanol)	258	57.5	Thin layer (III)	150	48.2
$^{14}\text{CO}_2$ (263 μC)	Thin layer (I)	75	115	Grade III alumina column	143	23.7	Thin layer (III)	112	16.5
	Thin layer (I)	58	115	Thin layer (III) (quinone)	82	16.0			

* The thin-layer systems used were: (I) benzene-light petroleum (2:3, v/v); (II) benzene-chloroform (1:1, v/v); (III) ethyl acetate-benzene (3:22, v/v).

The occurrence of the free alcohols, solanesol in leaves of tobacco (Kofler *et al.* 1959) and isodecaprenol ('spadicol') in the spadix of *Arum maculatum* (Hemming, Morton & Pennock, 1963), can be taken as evidence that the plant is capable of synthesizing independently of the nucleus units of the required size and configuration for quinone formation.

The next step in quinone formation would be the coupling of the alcohol pyrophosphate with either (a) the completely or nearly completely formed quinone nucleus, or (b) some early nuclear precursor; the coupling would occur between the reduced form of the quinone nucleus, i.e. quinol in (a) (Lynen, 1961), or some phenolic compound in (b). The first proposal (a), although popular for some time, has after numerous investigations with a variety of organisms and postulated ring precursors still no experimental basis (see e.g. Glover, 1965; Threlfall, 1967), except for two reports by Martius and co-workers that claimed to have shown that [methyl 6-³H]ubiquinone-0 is incorporated into ubiquinone in rat tissues (Stoffel & Martius, 1960) and by liver mitochondrial preparations from chick and rat (Schieffer & Martius, 1960).

The second route (b) has found experimental support mainly through the efforts of Rudney and co-workers. These workers have shown that *p*-hydroxybenzoic acid, a compound that has proved to be an effective precursor to the ubiquinone ring in a variety of organisms, e.g. *Rhodospirillum rubrum*, *Azotobacter vinelandii*, rat (Parson & Rudney, 1964), *Tetrahymena pyriformis* (Miller, 1965), baker's yeast (Spiller, 1966) and higher plants (Whistance, Threlfall & Goodwin, 1966), in *R. rubrum* gives rise to a compound 2-decaprenylphenol (Olsen *et al.* 1965), presumably by condensation followed by decarboxylation with decaprenyl pyrophosphate, which is then converted into ubiquinone-10 (Parson & Rudney, 1965) via 2-decaprenyl-6-hydroxyphenol and 2-decaprenyl-6-methoxyphenol (Olsen, Daves, Moore, Folkers & Rudney, 1966); the subsequent sequence of hydroxylation, *O*-methylation and *C*-methylation reactions is unknown.

The synthesis of the phytol side chain of phyloquinone, tocopherols and tocopherolquinone is no less obscure (since tocopherolquinones are probably metabolic oxidation products of the corresponding tocopherols it is only necessary to consider formation of the latter compounds). There would appear to be two choices. First, phytol pyrophosphate could be formed by partial reduction of geranylgeranyl pyrophosphate and this could then couple with the appropriate nuclear precursor. Alternatively, geranylgeranyl pyrophosphate could couple with the nuclear precursors and then undergo partial saturation to form phyloquinone, and

partial saturation and cyclization to form tocopherols. The presence of tocotrienols, which would seem to be ideal precursors for tocopherol formation, in various plant tissue, e.g. seed oils (Pennock, Hemming & Kerr, 1964) and *Hevea latex* (Dunphy, Whittle, Pennock & Morton, 1965), suggests that the latter pathway is the favoured one and that cyclization occurs before saturation of the side chain. In view of the occurrence of the various tocotrienols it might seem reasonable to expect to find, under suitable conditions, menaquinone-4 (i.e. menaquinone with 20 carbon atoms in the side chain) and a chlorophyll with a geranylgeranyl side chain.

The second part of the investigation was concerned with determining whether, as predicted by Goodwin & Mercer (1963), the prenyl side chain of the quinones localized within the chloroplast are biosynthesized there and whether the side chain of the extraplastidically located ubiquinone is formed elsewhere within the cell.

Scheme 2 outlines the possible relationship between the biosynthetic pathways involved in terpenoid formation in the photosynthetic cell and also makes clear the rationale behind the experimental approach used. Considering case (A) first, since this is the simplest, it is apparent that whether the label comes from ¹⁴CO₂ or from [2-¹⁴C]MVA the relative incorporation into the sterols, ubiquinone-9, β -carotene, phyloquinone, plastoquinone, α -tocopherolquinone and α -tocopherol will be the same. In view of the large synthesis of plastoquinone, α -tocopherolquinone, β -carotene and also to a smaller extent of phyloquinone and ubiquinone-9 (although ubiquinone-9 shows no net synthesis over the experimental period, after excision the amount falls and is then restored: this means in effect that appreciable synthesis of ubiquinone takes place), these compounds would be expected to have a much higher specific activity than the phytosterols.

In case (B) we have two pathways for terpenoid biosynthesis: one is in the chloroplast for the synthesis of the plastidic terpenoids (β -carotene, phyloquinone, plastoquinone, α -tocopherolquinone and α -tocopherol) and the other is outside the chloroplast for the formation of the extraplastidic terpenoids (phytosterols and ubiquinone-9). This means that there must be a segregation of enzymes, some of which are common to both pathways and others which are unique to one or other of the pathways. Clearly, if there are no physical factors determining the two sites of synthesis, i.e. the chloroplast membrane provides no barrier to MVA or other intermediates of terpenoid biosynthesis, then the labelling patterns from [2-¹⁴C]MVA and ¹⁴CO₂ will be identical with that in (A), and two pathways will be experimentally undetectable. However, if, as suggested by Goodwin, (1958) and

(A) Common biosynthetic pathway

(B) Two pathways differentiated by substrate availability

Scheme 2. Relationship between administered substrates and the biosynthetic sites of terpenoid quinones (prenyl portions) and related compounds in etiolated maize shoots exposed to light. The squares represent the pool sizes (not to scale) of sterol, ubiquinone and plastoquinone in the maize shoot at the end of the 'greening up' period. The squares are subdivided into (i) black areas, which represent the measurable incremental synthesis of the compound during the experiment, and (ii) white areas, which represent the original endogenous material.

verified experimentally by Rogers, Shah & Goodwin (1966), the chloroplast membrane is impermeable or partly permeable to $[2-^{14}\text{C}]\text{MVA}$ (and later intermediates of terpenoid biosynthesis), a different labelling pattern would be obtained for the two precursors. Thus $[2-^{14}\text{C}]\text{MVA}$ would be readily incorporated into the phytosterols and prenyl side chain of ubiquinone but only poorly into β -carotene and the prenyl side chains of phylloquinone, plastoquinone, α -tocopherolquinone and chlorophyll. On the other hand, $^{14}\text{CO}_2$ would, after fixation, readily enter the chloroplastidic terpenoid biosynthetic sequence. It is possible that $^{14}\text{CO}_2$ -fixation products suitable for terpenoid synthesis would be made available to the extraplasmidic site just as rapidly as to the intraplasmidic site. This would mean in effect that the labelling pattern from $^{14}\text{CO}_2$ would be as expected for a common biosynthetic site, i.e. the pattern predicted for (A). However,

experimentally the two substrates would still give different labelling patterns.

Table 3 summarizes the results for the incorporation of ^{14}C from the two substrates into plastoquinone and phytosterols of maize and barley shoots. The results of a fuller investigation into the synthesis of the solanesol-containing quinones, ubiquinone and plastoquinone, by maize shoots are given in Table 9. Table 9 also records the measurable synthesis of the various components that occurred under these experimental conditions. The comparisons between the various components are based solely on specific activities expressed in terms of actual weight of the compounds under investigation and no allowance is made for non-terpenoid portions of the molecules, e.g. the quinone ring. The refinement of relating activities to the portions of the molecules concerned, e.g. prenyl side chains of ubiquinone and plastoquinone or counts/g. atom of

Table 9. Incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{mevalonic acid}$ and $^{14}\text{CO}_2$ into plastoquinone, ubiquinone-9, β -carotene and unesterified 3β -hydroxy sterols by illuminated excised etiolated maize shoots

The Table summarizes the results obtained in Expt. II (Tables 4, 5 and 6) for the incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{MVA}$ and $^{14}\text{CO}_2$ into plastoquinone, ubiquinone-9, β -carotene and unesterified 3β -hydroxy sterols on illumination for 24 hr. of excised etiolated maize shoots.

Terpenoid	Increase in amount of terpenoid during expt.	$[2\text{-}^{14}\text{C}]\text{MVA}$ ($6\mu\text{C}/100$ shoots)			$^{14}\text{CO}_2$ ($200\mu\text{C}/100$ shoots)		
		Amount at end of expt. (mg./100 shoots)	$10^{-3} \times ^{14}\text{C}$ activity (counts/min.)	$10^{-3} \times \text{Sp.}$ activity (counts/min./mg.)	Amount at end of expt. (mg./100 shoots)	$10^{-3} \times ^{14}\text{C}$ activity (counts/min.)	$10^{-3} \times \text{Sp.}$ activity (counts/min./mg.)
Chloroplastidic							
Plastoquinone	4-fold	0.785	32	40.7	0.860	155	180
β -Carotene	10-20-fold	0.306	15	47.7	0.680	160	236
Extraplastidic							
Ubiquinone	2-fold?*	0.260*†	129	495	0.260*†	6.5	24.8
3β -Hydroxy sterols	0	23	2210	96	25	98	3.9

* Griffiths *et al.* (1967) have shown that, although there is no net change in the amount of ubiquinone under these experimental conditions, the amount does fall and then rise during the early period of illumination.

† Estimated from previous experiments (Griffiths *et al.* 1967).

^{14}C , was not considered necessary, since the differences between the various compounds are sufficient to allow a clear-cut interpretation of the results.

It is seen clearly that two patterns of incorporation exist. Thus $[2\text{-}^{14}\text{C}]\text{MVA}$ is effectively incorporated into ubiquinone (only shown for maize) and, if the size of the sterol pool is borne in mind, the 3β -hydroxy sterols. On the other hand, the incorporation into plastoquinone and β -carotene, if the large incremental synthesis of these compounds is taken into consideration, is poor. $^{14}\text{CO}_2$ shows the reverse behaviour, i.e. the radioactivity is effectively incorporated into plastoquinone and β -carotene. These results are compatible with the synthesis of the plastoquinone side chain occurring within the chloroplast and the ubiquinone-9 side chain outside the chloroplast. It is noteworthy that the prenyl side chain of these two quinones, which are the same length, can be synthesized at different sites within the plant cell.

The results obtained for the phytol-containing components were not as clear-cut (Table 10). Phylloquinone, in agreement with its chloroplastidic location, behaved in the experiments with the two substrates in a manner similar to β -carotene and plastoquinone. However, α -tocopherolquinone and α -tocopherol (both of which are localized in the chloroplast) gave labelling patterns that are not readily interpretable. Thus, considering the large incremental synthesis of α -tocopherolquinone (and, if α -tocopherol is metabolically related to α -tocopherolquinone, the total tocopherol pool) that has taken place under these experimental conditions, it

is apparent that this compound does not give either the β -carotene pattern, i.e. highly labelled from $^{14}\text{CO}_2$, or ubiquinone pattern (Table 9), i.e. highly labelled from $[2\text{-}^{14}\text{C}]\text{MVA}$. This result suggests that in maize shoots there is some unidentified tocopherol precursor that under the conditions of our experiments only required minor changes, such as methylation of the ring and saturation of the side chain, to form α -tocopherol, which would give α -tocopherolquinone on oxidation.

One objection that can be levelled against these experiments is that the results are being interpreted on a cellular basis, when they may in fact only be a reflection of the physiology of the excised shoots. Thus under the experimental conditions employed it is possible that $[2\text{-}^{14}\text{C}]\text{MVA}$ would be made available to the cells at the base of the shoot, where phytosterol and ubiquinone synthesis will probably outweigh β -carotene and plastoquinone synthesis, before the cells in the regions where active chloroplast maturation with concomitant β -carotene, plastoquinone, phylloquinone and α -tocopherolquinone synthesis is taking place. On gross analysis of the shoots the highly radioactive phytosterols from the base of the shoot would raise the specific activity of the phytosterols from the photosynthetic regions. Again, during the greening-up procedure before synthesis of the chloroplastidic components gets under way $[2\text{-}^{14}\text{C}]\text{MVA}$ could be incorporated into the sterols to a much greater extent than into the plastoquinone etc., giving in effect the picture obtained. Similarly, for $^{14}\text{CO}_2$ it might be expected that phytosterols in the lower region of the shoot would be relatively unradioactive and would dilute

Table 10. Incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{mevalonic acid}$ and $^{14}\text{CO}_2$ into phyloquinone, α -tocopherol, α -tocopherolquinone, β -carotene and unesterified 3β -hydroxy sterols by illuminated excised etiolated maize shoots

The Table summarizes the results obtained in Expt. IV (Table 8) for the incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{MVA}$ and $^{14}\text{CO}_2$ into phyloquinone, α -tocopherol, α -tocopherolquinone, β -carotene and unesterified 3β -hydroxy sterols on illumination for 24 hr. of excised etiolated maize shoots.

Terpenoid	Increase in amount of terpenoid during expt.	$[2\text{-}^{14}\text{C}]\text{MVA}$ ($6\mu\text{C}/100$ shoots)			$^{14}\text{CO}_2$ ($263\mu\text{C}/100$ shoots)		
		Amount at end of expt. (mg./100 shoots)	$10^{-3} \times ^{14}\text{C}$ activity (counts/min.)	$10^{-3} \times \text{Sp.}$ activity (counts/min./mg.)	Amount at end of expt. (mg./100 shoots)	$10^{-3} \times ^{14}\text{C}$ activity (counts/min.)	$10^{-3} \times \text{Sp.}$ activity (counts/min./mg.)
Chloroplastidic							
Phyloquinone	2-fold	0.112	2.96	26.4	0.92	106	115
α -Tocopherol†	0	0.226	5.99	26.5	0.258	4.13	16
α -Tocopherolquinone†	All formed	0.400*	1.42	3.8	0.400*	6.8	17
β -Carotene	10-fold	0.300	11.1	37	0.390	81.9	210
Extraplastidic							
3β -Hydroxy sterols	0	20*	2040	102	20*	82	4.1

* Estimated values.

† The purity of these compounds is not certain.

out the radioactivity in the phytosterols from cells containing chloroplasts and thus possibly labelled from $^{14}\text{CO}_2$ earlier. If in our experiments all the conditions above were true then it follows that, after making suitable allowances for them, the labelling patterns from $^{14}\text{CO}_2$ and $[2\text{-}^{14}\text{C}]\text{MVA}$ would be identical within the photosynthetic cell and our deductions as to the various sites of synthesis would be valueless.

The time-course experiment (Fig. 4 and Table 4) with $[2\text{-}^{14}\text{C}]\text{MVA}$ showed that $[2\text{-}^{14}\text{C}]\text{MVA}$ is made available to the plastoquinone-synthesizing sites from the beginning of the experiment, and that during the early stages of the experiments, before it is even possible to demonstrate a net synthesis of plastoquinone, label is being incorporated into this compound. Further, on analysing various segments of maize shoots that had been exposed to $[2\text{-}^{14}\text{C}]\text{MVA}$ or $^{14}\text{CO}_2$, it was shown that, although there was a slight gradient of radioactivity into the sterols with $[2\text{-}^{14}\text{C}]\text{MVA}$ as substrate (Table 7), gross analysis of the tissues provided a fair reflection of the situation pertaining to the more highly developed photosynthetic regions. $^{14}\text{CO}_2$ gave essentially a similar result. It is noteworthy that in the $^{14}\text{CO}_2$ experiment the sterols from the lower regions of the shoot have specific activities similar to those of the sterols from the higher more-photosynthetic regions, showing that photosynthetic fixation products are made available to the sterol-synthesizing sites in the lower regions of the shoot as rapidly as to sites in the same cells as the chloroplasts.

Thus all the results obtained are compatible with the view that in maize (and barley) shoots the prenyl side chain of phyloquinone and plastoquinone, along with β -carotene (Goodwin, 1958; Treharne *et al.* 1966) and the phytol side chain of chlorophyll (Mercer & Goodwin, 1962), are biosynthesized within the chloroplast, whereas the prenyl side chain of ubiquinone-9, along with the phytosterols, is biosynthesized elsewhere within the cell. No evidence was forthcoming as to the site of α -tocopherol and α -tocopherolquinone formation. Recent investigations suggest that in fact the entire molecules of the chloroplast-located terpenoid quinones and related compounds may be elaborated within this organelle (Threlfall & Griffiths, 1967; Threlfall, 1967; G. R. Whistance, unpublished work).

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