Nature, Intracellular Distribution and Formation of Terpenoid Quinones in Maize and Barley Shoots

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1. Maize and barley shoots have been shown to contain phyloquinone, plastoquinone, α -tocopherol (and γ -tocopherol in maize), α -tocopherolquinone and ubiquinone-9. 2. No solanesol was detected in any tissue examined. 3. In maize shoots plastoquinone and α -tocopherolquinone were localized in the chloroplast; ubiquinone was in the mitochondria. 4. Etiolated (dark-grown) shoots contained smaller amounts of phyloquinone and plastoquinone; α -tocopherolquinone was entirely absent; ubiquinone and α -tocopherol concentrations were unaffected. 5. On illumination of etiolated shoots the chloroplastidic quinones phyloquinone, plastoquinone and α -tocopherolquinone were synthesized in step with chloroplast development. α -Tocopherolquinone was not formed at the immediate expense of α -tocopherol.

The aims of the present series of investigations into the factors governing the biosynthesis of phytoquinones and a review of the state of knowledge at the inception of these studies have been given by Threlfall & Goodwin (1967). We now report on the nature, intracellular distribution and formation of phytoquinones and related compounds in young maize and barley shoots in relationship to chloroplast development.

The choice of these tissues was governed by the fact that young seedlings can be grown under artificial conditions and are metabolically very active. Of special relevance to the present investigation are the studies of Goodwin (1958) and Mercer & Goodwin (1962) on the biosynthesis of β -carotene and the phytol side chain of chlorophyll.

The quinone content of maize and barley has not before been the subject of intensive investigation. Crane (1959) reported briefly on the plastoquinone and ubiquinone contents of 7-day-old maize seedlings; the roots and shoots of *Zea mays* var. Golden Glow were found to contain 0.034 and 0.05 mg. of ubiquinone/g. dry wt. respectively; the corresponding values for plastoquinone were 0.018 and 0.082 mg./g. dry wt. The quinones were estimated in the same chromatographic fraction and no attempt was made to identify the homologues present.

Green (1958) demonstrated that, in addition to

α -tocopherol, maize seedlings contained γ -tocopherol and barley seedlings β -tocopherol, the total and relative amounts of which were governed by the age of the plant.

The results of the present investigation have already been reported briefly (Griffiths, Threlfall & Goodwin, 1964; Threlfall & Griffiths, 1966).

EXPERIMENTAL

Biological methods

Biological material. Maize (*Zea mays* var. South African White Horse Tooth) seeds were purchased from Gunsons (Seeds) Ltd., London, E.C. 3. Barley (*Hordeum sativum* Cb763 var. Proctor) seeds were a gift from the Welsh Plant Breeding Station, Aberystwyth.

Cultivation of maize seedlings. (i) Etiolated (dark-grown). Seeds were soaked in water in the dark for 24 hr. and then sown in large aluminium trays (150 seeds/20 in. $\times$ 14 in. $\times$ 3 in. tray or 500 seeds/36 in. $\times$ 22 in. $\times$ 3 in. tray) containing a sterile soil mixture (John Innes no. 1) to a depth of 2 in. Then, after a liberal watering, the seedlings were allowed to germinate for 6 days inside light-proof cupboards maintained at 26–28°. Over this period no further watering of the seedlings was required so that their germination in darkness was undisturbed.

At the end of 6 days the seedlings, which by this time were about 20 cm. high (the blade being about 15 cm. high), showing no secondary growth and completely devoid of any green colour, were removed from the cupboard in green light and excised at the node. The blades were taken for analysis.

(ii) Green (light-grown). Green seedlings were grown in essentially the same manner as the etiolated plants, except that germination was allowed to proceed under continuous illumination provided by a battery of 60 W tungsten lamps

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with reflectors (4 lamps/20 in. $\times$ 14 in. tray and 6 lamps/36 in. $\times$ 22 in. tray) held 30 cm. above the surface of the soil. Plants grown under these conditions normally reached a height of about 15 cm. in 6 days. The shoots, for analysis, were excised at soil level.

Cultivation of barley seedlings. Barley seedlings were grown under similar conditions to those described for maize except that the seeds were sown without prior soaking in heavily watered soil (100 seeds/36 in. $\times$ 22 in. $\times$ 3 in. tray) and allowed to germinate at a slightly lower temperature (24°). The etiolated barley after 6 days' germination tended to be shorter (about 12 cm.) and stood more erect than the etiolated maize seedlings, i.e. there was no excessive shoot growth. The green shoots were again slightly shorter than the corresponding etiolated shoots. The seedlings in both cases were excised at soil level and the blades taken for analysis.

Exposure of etiolated plants to light. (i) Intact seedlings. The tray containing the etiolated 6-day-old seedlings was removed from the dark cupboard and exposed for the required time-interval to the same lighting conditions used for the growth of green shoots.

(ii) Excised shoots. The shoots were excised at the node for maize or at soil level for barley, collected together into bundles of 100 shoots and the cut ends immersed in a shallow glass beaker (of sufficient height and diameter to support the shoots without cramping) containing water to a depth of 2 cm. The shoots were then illuminated as for the intact seedlings; during the greening-up process the depth of water in the beakers was kept at 2 cm.

Analytical methods

Fractionation of green maize shoots. Maize seedlings were germinated with continuous illumination (see above). After 6 days, about 300 seedlings were harvested and the leaf blades excised and cut into small pieces. This material (120 g.) was homogenized in two equal batches in a total volume of 160 ml. of NaCl buffer (0.35 M-NaCl-0.01 M-KH₂PO₄-0.1 M-EDTA, adjusted to pH 7.4; Stumpf & James, 1963). Homogenization was carried out for 30 sec. in an MSE Ato-Mix blender precooled to 0°, and all subsequent operations were carried out at this temperature. The brei was filtered through eight layers of cheesecloth (Holm-Hansen, Pon, Nishido, Moses & Calvin, 1959), which removed most of the fibrous tissue and cell debris. The resulting dark-green suspension was centrifuged in 50 ml. buckets (MSE Minor bench centrifuge) at 140 g for 5 min.; the sediment of cell debris was discarded.

The supernatant was transferred to 50 ml. polypropylene tubes and centrifuged at 5000 rev./min. (3000 g) for 15 min. in the Servall Superspeed model RC-2 automatic refrigerated centrifuge with SS-34 head. This procedure gave a dark-green sediment comprising chloroplasts, chloroplast fragments and some mitochondria; for description purposes the fraction was designated 'chloroplasts'.

The 'mitochondria' fraction (20 000 g) was obtained as a pale-green sediment by centrifuging the supernatant in 50 ml. stainless-steel tubes at 13 000 rev./min. for 15 min. in the Servall Superspeed model RC-2 centrifuge with SS-34 head. This fraction contains in addition to mitochondria some chloroplast fragments.

The resultant brown supernatant (the browning was due to the action of polyphenol oxidases), which at this stage

contained the microsomes and other particles of similar size, was finally centrifuged in a Beckmann Spinco model L preparative centrifuge at 150 000 g for 90 min. The supernatant was decanted, leaving the 'microsome' fraction on the bottom and side of the tubes.

Each sedimented fraction, i.e. 'chloroplasts', 'mitochondria' and 'microsomes', was extracted by boiling until colourless with ethanol-ether (1:1, v/v) mixture (three times). The combined extracts were collected in a separating funnel, washed four times with water to remove the ethanol and then dried over anhydrous Na₂SO₄. After filtering to remove the Na₂SO₄, samples of the extracts were taken for chlorophyll assay (see below) and the remainder was evaporated to dryness under a stream of N₂. Lipid material was extracted from the supernatant by boiling with ethanol (1 vol.), diluting with water and extracting with ether; the extract was then evaporated to dryness as above.

Extraction of a quinone-containing lipid fraction from plant material. After a thorough investigation of the methods available for the extraction of a quinone-containing fraction from plant materials the method finally adopted for routine use was a total lipid extraction of freshly harvested biological material.

The excised blades from the seedlings were rinsed with water, damp-dried between layers of filter paper and weighed. Samples, usually ten shoots, were normally taken at this stage for dry-weight determination by drying to constant weight in an oven at 98°. The shoots (100) were then cut up into a 500 ml. tapered beaker, containing just sufficient A.R. acetone to cover the plant material, and comminuted for two 1 min. periods with an Ultra-Turrax gun homogenizer. The dark-green (yellow with etiolated shoots) acetone extract was separated from the debris by suction through a sintered glass funnel into a separating funnel. The cell-material remaining in the sinter was then re-extracted a further two times with acetone and once with peroxide-free ether. After this treatment the residue was free of colour.

To the combined extracts in the separating funnel, peroxide-free ether ($\frac{1}{3}$ vol.) was added and, after shaking to mix the acetone and ether layers completely, the acetone was washed out by the careful addition of water down the side of the separating funnel. Three washes, of 1-2 vol. each, with cold water and one with warm water (50°) were usually sufficient to remove all traces of acetone from the extract; the colourless aqueous-acetone layer after each washing was discarded.

The ethereal extract was then dried over anhydrous Na₂SO₄ (A.R.) for 30 min. and filtered through a sintered-glass funnel. Normally, at this stage the extract was made up to a known volume and samples were taken for chlorophyll assay (see below). The extract was then concentrated by distillation, transferred to a tared conical flask and the solvent removed by evaporation under N₂. Last traces of solvent were removed by desiccation *in vacuo* and the conical flask was reweighed.

The extraction and all subsequent manipulations involving quinone-containing fractions were carried out without exposure of the sample to excessive heat or light. All fractions were stored at -20° until required.

Chromatography of the lipid fraction on alumina. Preliminary fractionation of the lipid extract was carried out by chromatography on a column of Brockmann grade III acid-washed alumina (Woelm anionotropic), developed by

stepwise elution with 0.25%, 1%, 3%, 5%, 8%, 12%, and 20% E/P* (Threlfall & Goodwin, 1967).

The fractions were evaporated to dryness (under N_2) in tared vessels, desiccated to remove the last traces of solvent, weighed and the u.v. or visible spectra (or both) determined in a suitable solvent. Table 1 lists the elution pattern for the various components examined in these experiments. Under these experimental conditions the various compounds with which we were concerned are eluted completely by the appropriate fraction and no overlap between fractions takes place.

Some of the fractions obtained from the preliminary alumina-column chromatography were purified further on columns of Brockmann grade II acid-washed alumina (Woelm anionotropic), for example, the 0.25%-E/P fraction, which contains phyloquinone, cannot be estimated spectrophotometrically in the presence of β -carotene. Irrespective of the weight of the fraction a column of the same dimensions as the column used for preliminary chromatography was used. With the 0.25%-E/P fraction the column was developed with 1% and 2% E/P, the phyloquinone being eluted in the 2%-E/P fraction.

Thin-layer chromatography. Qualitative and quantitative thin-layer chromatography was carried out on 250 μ -thick layers of Kieselgel G (E. Merck A.-G., Darmstadt, Germany). The systems and reagent sprays used were as reported by Threlfall & Goodwin (1967). In some of the experimental work reported here use was made of thin layers impregnated with the dye Rhodamine 6G (Avigan, Goodman & Steinberg, 1963). On examination of the developed plates under u.v. light (Hanovia Chromatolite lamp) all u.v.-absorbing materials, e.g. quinones and chromanols, appear as dark-blue spots against a yellow background. Phytosterols, squalene and solanesol appear as red spots against a yellow background. Thus a ready differentiation can be made between these compounds and, most important in quantitative work, the precise location of the compound under investigation is obtained. In quantitative work, the dye and adsorbent are readily separated from the resolved lipids by our usual elution techniques (Threlfall & Goodwin, 1967). Absolutely dry ether must be used otherwise the dye will be eluted with the lipid by the traces of water present.

Paper chromatography. This was carried out as described by Threlfall & Goodwin (1967).

Quantitative estimation of components. The amounts of β -carotene, plastoquinone, ubiquinone and α -tocopherol-quinone present in a tissue were determined spectroscopically (Threlfall & Goodwin, 1967) on the appropriate column fractions from the chromatography of the lipid fraction. Phyloquinone was determined spectroscopically (Threlfall & Goodwin, 1967) on the fraction obtained by rechromatographing the 0.25%-E/P fraction on alumina (see above).

Tocopherols were estimated spectrophotometrically after quantitative chromatography of the appropriate column fractions (3% E/P for α -tocopherol; 5% E/P for γ -tocopherol on thin-layers of Kieselgel G (Pennock, Neiss & Mahler, 1962).

Chlorophyll was determined as chlorophyll ($a+b$) by taking a suitable sample of the lipid extract (see above), removing the solvent by vacuum desiccation, making up to a

suitable volume with acetone-water (4:1, v/v) and measuring the extinction at 645 and 663 m μ (Arnon, 1949).

Spectroscopy. The u.v. and visible spectra were determined with the Perkins-Elmer 137 Uvicord spectrophotometer; all absorption maxima and reductions at specific wavelengths were checked on the Unicam SP.500 spectrophotometer.

The i.r. spectra were determined as solvent-free films or KBr disks with the Perkin-Elmer 137E Infracord spectrophotometer.

Solvents. Light petroleum (b.p. 40–60°), benzene (A.R.) and diethyl ether (A.R.) were dried over sodium wire and redistilled; the diethyl ether was distilled over reduced iron immediately before use.

Ethanol and cyclohexane were of spectroscopic grade.

Presentation of results

One of the difficulties encountered during the present investigations has been the selection of a suitable reference standard for experiments of a comparative nature. Various authors have in the past expressed quinone concentrations in relation to chlorophyll concentrations, fresh weight and dry weight. In the present work certain difficulties became apparent during attempts to relate quinone values to any of these parameters. Clearly chlorophyll concentrations could not be used as a basis for the expression of any results that involved analysis of etiolated tissues. Similarly, although fresh weight and dry weight in some experiments provided a satisfactory method of comparison, in others significant weight changes, which were not accompanied by gross morphological changes, took place and this led to erroneous values; for example, in experiments where excised etiolated shoots were exposed to light a marked decrease in dry weight of the shoots occurred during the experiments even though the overall dimensions of the shoots remained unaltered.

The method of expressing data finally adopted, and the one found to be the most reliable, was to base results on plant numbers. With the introduction of large cultivation trays in which large numbers of seeds could be germinated under identical conditions growth of the seedlings was very uniform with only very slight interseedling variations under any one set of particular experimental conditions.

RESULTS AND DISCUSSION

Quinones and chromanols in green and etiolated maize and barley shoots. Griffiths (1965) demonstrated that the extraction procedure and subsequent purification step, i.e. column chromatography used in the present investigation, leads to quantitative recovery of all quinones examined. It was observed during the present work that maize shoots contained, in addition to plastoquinone and ubiquinone, two other quinones that were tentatively identified as phyloquinone and α -tocopherol-quinone; further, the crude ubiquinone fraction behaved as ubiquinone-9 rather than the ubiquinone-10 form (the numerals refer to numbers of isoprene units/mol.) reported by Crane (1959).

To characterize the quinones more fully several moderately large-scale analyses of green and

* Abbreviation: E/P, solution of diethyl ether in light petroleum (b.p. 40–60°).

Table 1. *Initial chromatography of total lipid extract from green maize shoots*

The 215 7-day-old shoots (wet wt. 195g., dry wt. 14.85g.), average height 18cm., were extracted in the normal manner, as described in the text. The extract, 1.081 g. of a black to dark-green oil containing 149.5mg. of chlorophyll, was chromatographed on a column of 100g. of Brockmann grade III alumina. Seven 1l. fractions of light petroleum (b.p. 40–60°) containing increasing amounts of ether were collected.

Fraction	Eluent	Wt. (mg.)	Appearance of fraction and comments
1	0.25% E/P	26.2	Dark-brown oil; squalene, β -carotene (1.125mg.) and phyloquinone (178 μ g.) (the u.v. spectrum of the last compound is masked at this stage) present
2	1% E/P	8.4	Lemon-yellow oil; plastoquinone (3.365mg.) and sterol esters present
3	3% E/P	4.8	Dark-red oil; fraction contains α -tocopherol
4	5% E/P	28.2	Orange waxy solid; contains ubiquinone (592 μ g.), γ -tocopherol, long-chain saturated alcohol and various intermediates of sterol biosynthesis
5	8% E/P	16.3	Yellow solid
6	12% E/P	42.9	Yellowish-white solid; nearly the whole of the fraction is made up of β -sitosterol, stigmasterol and campesterol
7	20% E/P	9.4	Dark-yellow oil; tocopherolquinone (1.258mg.) present

Recovery: 136.3 (12.6%)

etiolated maize and barley shoots were carried out. At the same time the shoots were examined for the presence of tocopherols and the nonaprenyl alcohol solanesol (Rowland, Latimer & Giles, 1965a,b; Kofler *et al.* 1959).

Table 1 gives the pertinent details of the extraction, initial chromatography and relevant u.v. spectra of the fractions from the analysis carried out on 7-day-old green maize shoots. The results for green barley and etiolated maize and barley shoots were similar with regard to the general nature and distribution of lipid between the various fractions, the most notable differences being the absence of chlorophyll and α -tocopherolquinone from etiolated tissues.

Fraction 1 (0.25% E/P) was in all cases a heavy oily fraction of a pale-yellow colour from the etiolated plant lipid and a dark-brown colour from the green plant. The visible spectrum of the fraction determined in light petroleum (b.p. 40–60°) showed absorption maxima at positions identical with those of β -carotene, i.e. $\lambda_{\max}$ 425, 451 and 482 m μ . On chromatography of samples on thin layers developed with benzene–light petroleum (b.p. 40–60°) (1:19, v/v), the coloured component in all cases ran with and was not separated from β -carotene (R_f 0.6).

The u.v. spectra of these fractions, which contain phyloquinone, exhibit only irrelevant absorption. To characterize the vitamin the remainder of the fraction was rechromatographed on a column of Brockmann grade II alumina (see the Experimental section); 2% E/P eluted a pale-yellow oil, which, after determination of its u.v. spectrum (Fig. 1), was purified further by quantitative thin-

Fig. 1. Ultraviolet spectra of phyloquinone purified by column and thin-layer chromatography. —, Phyloquinone fraction (in 15ml. of cyclohexane) after chromatography of the 0.25%-E/P fraction on alumina; ---, phyloquinone fraction (in 10ml. of cyclohexane) after thin-layer chromatography.

layer chromatography [Kieselgel G with benzene–light petroleum (b.p. 40–60°) (2:3, v/v) as developing solvent] and a single leucomethylene blue-staining compound recovered (R_f 0.46). The u.v. spectrum of the recovered material (Fig. 1) was superimposable on one of authentic phyloquinone ($\lambda_{\max}$ 243, 249, 262 and 271 m μ). On chromatography in the two reversed-phase systems described

by Threlfall & Goodwin (1967) the sample behaved as authentic phyloquinone.

Fraction 2 (1% E/P) was a pale-yellow oil, the oxidized and reduced u.v. spectra of which were readily attributable to the presence of plastoquinone, i.e. $\lambda_{\max}$ (oxidized) at 255 m μ . It was found that plastoquinone accounted for as little as 3% of the weight of the fraction in etiolated barley, but up to 50% in green maize. The fraction was rechromatographed on Brockmann grade II alumina as just described for phyloquinone. Plastoquinone was eluted by 2% E/P and was separated from a contaminating carotenoid, present in the original fraction, which was strongly adsorbed on the column. Samples of the resulting much-purified plastoquinone ran with and could not be separated from the authentic plastoquinone during chromatography on reversed-phase paper or on thin-layer chromatography.

The purified plastoquinone samples from lipids of both etiolated and green maize were combined (about 3.8 mg.), taken up in hot ethanol (1 ml.) and crystallized overnight at room temperature. This gave 2.06 mg. of pale-yellow crystals having $E_{1\text{cm}}^{1\%}$ 230 at 254 m μ in light petroleum and m.p. 45° (authentic plastoquinone has $E_{1\text{cm}}^{1\%}$ 253 at 254 m μ and m.p. 47°). Although not demonstrated in this analysis it is now known that the plastoquinone fraction obtained from the second chromatography contains sterol esters (Threlfall, Griffiths & Goodwin 1967), and so it must be concluded that, since the correct m.p. and $E_{1\text{cm}}^{1\%}$ for plastoquinone were obtained in the above experiment, these esters do not co-crystallize with plastoquinone.

During subsequent experiments the plastoquinone fractions from maize and barley lipids were collected, combined in their respective groups, purified by quantitative thin-layer chromatography to remove sterol esters and then recrystallized several times from hot ethanol. In all cases the m.p., $E_{1\text{cm}}^{1\%}$ (when enough material was available) and i.r. spectra of the crystals were in agreement with the values and spectra obtained for authentic plastoquinone.

Fraction 3 (3% E/P) was a fraction the u.v. spectrum of which in ethanol showed no change on addition of sodium borohydride, indicating the absence of quinones. However, ill-defined absorption bands in the region of 295 m μ suggested the presence of tocopherols. On quantitative thin-layer chromatography [Kieselgel G with chloroform-benzene (1:1, v/v)] two Emmerie-Engel-staining compounds with R_f values 0.5 and 0.9 were recovered. The more-polar band was identified as α -tocopherol by using the procedures described by Threlfall & Goodwin (1967). The recovered less-polar band was a colourless oil having $\lambda_{\max}$ 246, 258 and 285 m μ in cyclohexane. No assignment could be

made to the chromophoric group responsible for the spectrum. This compound was only found in green and etiolated maize.

Fraction 4 (5% E/P) was a heavy insoluble fraction the spectrum of which in ethanol showed a broad ill-defined peak at 275 m μ , indicating the presence of ubiquinone. Thin-layer chromatography of a sample in the system just described, followed by spraying with Emmerie-Engel reagent, showed the presence of a single reducing spot with R_f identical with that of β - or γ -tocopherol.

Difficulty was encountered during attempts to solubilize this and other 5%-E/P fractions for quantitative thin-layer chromatography owing to the presence of a waxy white residue (the bulk of this fraction is composed of an unsaturated long-chain alcohol). The fraction was eventually taken up in acetone and with difficulty chromatographed on thin layers [Kieselgel G with benzene-chloroform (1:1, v/v)]. In this way a single leucomethylene blue-staining compound (R_f 0.5) was recovered. The spectra (oxidized and reduced) of the recovered sample were identical with those of authentic ubiquinone (cf. Fig. 4 in Threlfall & Goodwin, 1967).

The purified ubiquinone samples from maize and barley shoots were chromatographed along with authentic ubiquinone-10, ubiquinone-9, ubiquinone-8 and ubiquinone-7 in two reversed-phase systems that allow for the separation of ubiquinone homologues (Threlfall & Goodwin, 1967). In all cases the quinones behaved as the ubiquinone-9 form.

Fraction 5 (8% E/P) from all lipids showed no selective absorption when examined in the u.v. region. Addition of sodium borohydride to the fractions had no effect on the spectrum, indicating the absence of any quinonoid compounds. Any solanesol present in the tissues would have been eluted in these fractions, and in view of this all the fractions were chromatographed along with authentic solanesol on thin layers of Kieselgel G with methanol-benzene (1:99, v/v) as mobile phase. After development the plates were exposed to iodine vapour (Mercer, Davies & Goodwin, 1963); the solanesol showed up as a brown-staining spot (R_f 0.4); however, no spots were seen in the corresponding regions of the chromatographic fractions.

Fraction 6 (12% E/P) exhibited no selective absorption in the u.v. region and on treatment with sodium borohydride no spectral changes were observed. Griffiths (1965) has demonstrated that this fraction consists almost entirely of 3 β -hydroxysterols. Green barley was atypical in that it exhibited u.v. absorption at $\lambda_{\max}$ 260 m μ , and on treatment with sodium borohydride changes occurred consistent with the presence of trace amounts of a quinone, the chromatographic properties of which were similar to plastoquinone C (Threlfall, Griffiths

& Goodwin, 1965), although the extremely small amounts present are not consistent with the occurrence of the quinone.

Fraction 7 (20% E/P) contained a substance with a very intense bicuspid peak in the u.v. with $\lambda_{\max}$ 262 and 270 m μ in ethanol, suggesting the presence of α -tocopherolquinone. These peaks were abolished by the addition of sodium borohydride and replaced by another of lower intensity at 291 m μ . The fraction was purified further by quantitative thin-layer chromatography [Kieselgel G with ethyl acetate-benzene (3:22, v/v)] and a single leucomethylene blue-staining compound recovered (R_f 0.44). The recovered material had a u.v. spectrum identical with that of α -tocopherolquinone, and in all thin-layer-chromatographic systems listed by Threlfall *et al.* (1965) behaved as α -tocopherolquinone.

The quantitative results for this analysis and similar analyses carried out on green barley and etiolated maize and barley shoots are given in Table 2. The quinone concentrations of maize and barley shoots at different stages of maturity are given in Tables 4 and 5.

The results show that green maize and barley shoots contain phyloquinone, plastoquinone, α -tocopherolquinone and ubiquinone-9. Barley also contained a trace of a fifth quinone with chromatographic properties similar to those of plastoquinone C (Threlfall *et al.* 1965); however, apart from this no evidence could be found for the occurrence of the as yet incompletely characterized plastoquinone B (Kegel, Henninger & Crane, 1962), plastoquinone C and plastoquinone D (Henninger & Crane, 1963; Threlfall *et al.* 1965; Das, Lounasmaa, Tendile &

Lederer, 1965). The failure to detect these compounds cannot be ascribed to the inadequacy of the technique, since with the same analytical methods all three quinones have been isolated from other tissues (Griffiths, 1965). It must be concluded that in young maize shoots these compounds are either absent or occur in extremely small amounts.

The ubiquinone homologue in maize was found to be ubiquinone-9 rather than the ubiquinone-10 form reported by Crane (1959); however, on the basis of work carried out on other higher-plant tissues, Crane (1959) assumed that maize would contain only ubiquinone-10. Indirect evidence in support of our identification comes from the work of Page, Gale, Koniuszy & Folkers (1959), who found that corn oil (maize-seed oil) contains ubiquinone-9.

In agreement with the observations of Green (1958) α -tocopherol was found in both maize and barley shoots, and in maize a second tocopherol with chromatographic properties similar to β - or γ -tocopherol was also detected. On the basis of Green's (1958) work this would be γ -tocopherol. Barley was not examined for the presence of other tocopherols, although whole seedlings contain β -tocopherol in addition to α -tocopherol (Green, 1958).

Solanesol, a nonaprenyl alcohol, the pyrophosphate of which could give rise to the plastoquinone and ubiquinone-9 chains (Threlfall & Griffiths, 1966), was not detected in any tissue.

The concentrations of the plastidic quinones (phyloquinone, plastoquinone and α -tocopherolquinone) in maize and barley shoots are low when compared with other photosynthetic tissues such as leaves, but this is only to be expected from the

Table 2. *Quinone concentrations in green and etiolated maize and barley shoots*

The number of shoots taken for each analysis was 215 for maize and 600 for barley.

	Concn. in maize (7-day-old-shoots)				Concn. in barley (5-day-old-shoots)			
	(per 100 shoots)		(per g. dry wt.)		(per 100 shoots)		(per g. dry wt.)	
	Green	Etiolated	Green	Etiolated	Green	Etiolated	Green	Etiolated
Height (cm.)	18	22	—	—	8	13	—	—
Fresh wt. (g.)	91	110	13.1	14.1	5.21	8.71	8.68	11.3
Dry wt. (g.)	6.91	7.79	—	—	0.60	0.77	—	—
Lipid extract (mg.)	503	323	72.8	41.4	34.2	42.8	57.0	55.6
Chlorophyll (mg.)	69.6	0	10.1	0	3.89	0	6.47	0
β -Carotene (μ g.)	523*	149	77.4	19.2	70	Trace	117	Trace
Phylloquinone (μ g.)	83	74	12.2	9.5	Present	Present	Present	Present
Plastoquinone (μ g.)	1565	223	227	29	146	81	243	103
α -Tocopherolquinone† (μ g.)	585	0	86	0	24	0	40	0
Ubiquinone-9 (μ g.)	275	304	41	38	27	29	45	38

* Lower than expected (cf. Table 5).

† α -Tocopherol was present in all shoots examined; in addition, maize contains γ -tocopherol and barley contains β -tocopherol (Green, 1958).

fibrous nature of the tissue. The concentration of plastoquinone for 7-day-old maize shoots found by us is much higher than that originally reported by Crane (1959) (227 μg . as compared with 82 μg . of plastoquinone/g. dry wt.). It is possible that the preheating of the fresh material at 100° carried out by Crane (1959) resulted in some destruction. The ubiquinone values for this tissue were, however, in good agreement with those of Crane (1959). The concentrations of the chloroplastidic quinones relative to each other are similar to those found in other photosynthesis tissues, with plastoquinone the predominant form, followed by α -tocopherolquinone and phyloquinone (see Table 8 in Threlfall & Goodwin, 1967).

A comparison of green and etiolated shoots (Tables 2, 4 and 5) brought out some noteworthy facts. In the absence of light, maize and barley shoots contained lower amounts of phyloquinone, plastoquinone and β -carotene and no chlorophyll or α -tocopherolquinone. The presence of the 'plastidic' quinones phyloquinone and plastoquinone in etiolated tissues, thus demonstrating that formation of these compounds but not their concentration is independent of illumination, was not unexpected, since Dam & Glavind (1938) and Dam, Glavind & Nielson (1940) had previously shown that, although phyloquinone was synthesized by dark-grown seedlings of *Picea canadensis*, the concentration was significantly lower than in light-grown seedlings. However, in dark-germinated *Pisum sativum* no phyloquinone was formed at all. Similarly, Egger (1963) had reported that plastoquinone concentrations in etiolated maize seedlings are significantly lower than in the corresponding green seedlings. The high plastoquinone content in green shoots matches the photosynthetic activity of such tissues, a process in which this quinone appears to play an important role (Redfearn, 1965).

Ubiquinone was found in both etiolated and green shoots at approximately the same concentration, a result that could perhaps have been anticipated from the role this quinone plays in terminal respiration, a process that must occur in both green and etiolated tissues.

In view of the association of phyloquinone, plastoquinone and α -tocopherol with chloroplasts in green tissues and the occurrence of these components in etiolated and non-photosynthetic plant tissues, we are led to suggest that, in etiolated maize and barley shoots, these compounds are associated with the proplastids, structures that on illumination give rise to mature chloroplasts. From this it follows that the occurrence of plastoquinone in non-photosynthetic tissues such as corn roots (Crane, 1959), the spadix of *Arum maculatum* (Hemming, Morton & Pennock, 1963) and meristematic tissue of Paul's Scarlet Rose (Threlfall & Goodwin, 1963) is

indicative of the occurrence in these tissues of particles that are either potentially capable or have lost their ability of developing into chloroplasts (Crane, 1961; Threlfall & Griffiths, 1966).

Intracellular distribution of plastoquinone, α -tocopherolquinone and ubiquinone-9 in green maize shoots. Intracellular-distribution studies have shown that in photosynthetic tissues of higher plants phyloquinone, plastoquinone, α -tocopherolquinone and α -tocopherol are almost, if not entirely, associated with the chloroplast (Lichtenthaler & Calvin, 1964).

On the basis of limited studies so far carried out ubiquinone appears to be associated with the mitochondria (Crane, 1959; Threlfall & Goodwin, 1964), as it is in animal tissues (Crane, 1965).

Since the main intention of the experiment was to show the intra- or extra-plastidic localization of the various compounds under investigation, it was considered that rigorous purification of one particle, e.g. the chloroplast, would not provide the information required. It appeared that the best approach was to carry out a sedimentation-distribution study and then to compare the distribution of the components under investigation with that of compounds (chlorophyll and β -carotene) known to occur only in the chloroplast. Table 3 gives the results for such a study carried out on green maize shoots. The distribution of plastoquinone and α -tocopherolquinone compared with that of chlorophyll and β -carotene is clearly consistent with their occurrence in the chloroplast; ubiquinone, on the other hand, exhibits a pattern in keeping with its localization elsewhere in the plant cell, i.e. mitochondria.

Although the distribution of plastoquinone and α -tocopherolquinone parallels chlorophyll and β -carotene distribution, there are minor discrepancies in the percentage distribution. However, since there is some divergence between the controls, i.e. β -carotene fails to parallel chlorophyll exactly, these variations can probably be attributed to experimental error, or possibly to the predominance in a fraction of immature chloroplasts or chloroplast fragments having a composition slightly different from that of the intact or mature chloroplasts. After allowing for these variations the only result requiring comment is the occurrence in the supernatant of plastoquinone unaccompanied by chlorophyll or β -carotene. Threlfall & Goodwin (1964) and Stevenson, Hemming & Morton (1963) have observed plastoquinone in significant amounts in the soluble phase of *Euglena* cells and tobacco leaves for which two explanations have been proposed. Threlfall & Goodwin (1964) suggested that plastoquinone could have been leached out of the *Euglena* chloroplasts during ultrasonic treatment (the method used to disrupt the cells) or that the procedure may result in the liberation of

osmiophilic globules rich in plastoquinone, but containing no chlorophyll or β -carotene (Bailey & Whyborn, 1963). Alternatively, Stevenson *et al.* (1963) suggested that the plastoquinone in the supernatant of tobacco leaves may be derived from small underdeveloped plastids remaining in the supernatant under these conditions.

Effect of light on plastoquinone, α -tocopherolquinone and ubiquinone-9 synthesis by etiolated maize and barley shoots. Kay & Phinney (1956) had shown that the illumination of etiolated maize seedlings results within the first 24–48 hr. in a rapid synthesis of β -carotene. Goodwin (1958) further demonstrated that illumination of etiolated maize seedlings results in the formation of chloroplasts

from pre-existing proplastids, with the concomitant synthesis of chlorophyll and β -carotene within the developing organelles.

It was therefore decided to test whether synthesis of the chloroplastidic quinones phyloquinone, plastoquinone and α -tocopherolquinone by etiolated maize and barley seedlings could also be stimulated by white light during the process of chloroplast development. Information would also be gained on the effect of this process on the synthesis of the extrachloroplastidic ubiquinone-9.

The investigation was carried out with intact and excised shoots. The choice of the latter experimental system was governed by two considerations. First, excision of the shoots effectively prevents the

Table 3. *Intracellular distribution of chlorophyll, β -carotene, plastoquinone, α -tocopherolquinone and ubiquinone-9 in green maize shoots*

Experimental conditions are given in the text.

Fraction	Chlorophyll		β -Carotene		Plastoquinone		α -Tocopherolquinone		Ubi-quinone-9	
	(mg.)	(%)	(μ g.)	(%)	(μ g.)	(%)	(μ g.)	(%)	(μ g.)	(%)
3000g ('chloroplasts')	2.20	71.1	80	64.5	246	68.4	97	67.8	15	24.2
20000g ('mitochondria')	0.69	22.3	38	30.7	106	29.4	46	32.2	39	62.9
105000g ('microsomes')	0.21	6.6	6	4.8	0	0	0	0	8	12.9
Supernatant	0	0	0	0	8	2.2	0	0	0	0

Table 4. *Effect of light on the synthesis of plastoquinone, α -tocopherolquinone and ubiquinone-9 by excised etiolated maize shoots*

Maize seeds were germinated in the light or dark and the shoots analysed for quinone content at suitable time-intervals. After 5 days 300 dark-grown shoots were excised and exposed to continuous illumination; samples were taken for analysis after 0 hr., 3 hr., 6 hr., 12 hr. and 24 hr. exposure to light. The number of shoots taken for each analysis was 50.

Age (days)	Conditions of growth	Concn./100 shoots						
		Dry wt. (g./100 shoots)	Length of shoot (cm.)	Chlorophyll (mg.)	β -Carotene (μ g.)	Plastoquinone (μ g.)	α -Tocopherolquinone (μ g.)	Ubi-quinone-9 (μ g.)
4	Light	1.94	7	11.4	261	337	240	134
	Dark	3.54	10	0	8.6	57	0	60
6	Light	5.21	12	37.3	Lost	1970	510	230
	Dark	6.14	18	0	103	241	Trace	220
	Dark-grown shoots excised and exposed to light* for:							
	3 hr.		18	2.8	11.5	235	70	140
	40 hr.		18	23.7	420	942	413	275
	Dark-grown shoots exposed to light for 40 hr.†	8.57	19	54.7	1155	1900	460	Lost
8	Dark†	8.92	26	0	335	620	110	225

* See Fig. 2 for intermediate values.

† Secondary growth present.

contribution of other parts of the seedlings to the greening-up process, and confines the study to the potential of the developing chloroplast; secondly, excised shoots are necessary for experiments with radioactive terpenoid precursors, which are unable to penetrate the root systems of cereals but can enter the excised shoots (Goodwin, 1958).

(a) Intact shoots. Experiments with intact shoots were only carried out on maize seedlings, since the study was a pilot study for the excised shoots to be used later on. In a typical experiment 6-day-old dark-grown seedlings were exposed to continuous illumination for 36 hr. At the end of the period it was found that the plastoquinone concentration had risen from 243 to 1110 $\mu\text{g./g.}$ fresh wt., the ubiquinone concentration rising only slightly from 313 to 363 $\mu\text{g./g.}$ fresh wt. The values show clearly that exposure of the seedlings to light leads to a marked increase in the plastoquinone concentration, whereas the ubiquinone values are relatively unaffected.

(b) Excised shoots. Tables 4 and 5 and Figs. 2 and 3 summarize the effect of light on the synthesis of phytoquinones by excised etiolated maize and barley shoots. Also included in Tables 4 and 5 are results for the analysis of green and etiolated shoots at different stages of maturity carried out during this experiment.

In examining the effect of light on etiolated shoots consideration must be given to which plants

represent the true controls. Since excision leads to termination of growth the true controls are the etiolated shoots at the start of the experiment, because in the 6 day + 40 hr. shoots considerable shoot elongation has taken place (Table 4).

The results (Figs. 2 and 3) show that during the first 3 hr. the concentrations of β -carotene, plastoquinone and ubiquinone-9 in the shoots fall owing to photodestruction or enzymic destruction (or both) brought about by excision. After this, β -carotene, plastoquinone and α -tocopherolquinone (which is absent from etiolated shoots) are formed in step with chlorophyll and hence chloroplast development. Ubiquinone-9 is resynthesized until the concentration in the original etiolated shoot is attained. The synthesis of chloroplast components (chlorophyll, β -carotene, plastoquinone and α -tocopherolquinone) continues actively for about 24 hr. and then begins to fall away. This slowing up is probably a consequence of using excised shoots, since intact shoots exposed to light (growth of the shoot is inhibited to some extent under these conditions and so the results are comparable) were able to synthesize much greater amounts of chlorophyll, β -carotene, plastoquinone and α -tocopherolquinone during the same period of illumination (Tables 4 and 5). With maize some secondary growth of the intact shoots takes place and allowance should be made for this. The incremental synthesis of plastoquinone

Table 5. *Effect of light on the synthesis of plastoquinone, α -tocopherolquinone and ubiquinone-9 by excised etiolated barley shoots*

Barley seeds were germinated in the light or dark and the shoots analysed for quinone content at suitable time-intervals. After 5 days 2500 dark-grown shoots were excised and exposed to continuous illumination; samples were taken for analysis after 0 hr., 3 hr., 6 hr., 12 hr. and 24 hr. exposure to light. The number of shoots taken for each analysis was 500.

Age (days)	Conditions of growth	Dry wt. (g./100 shoots)	Length of shoot (cm.)	Concn./100 shoots				
				Chloro- phyll (mg.)	β - Carotene ($\mu\text{g.}$)	Plasto- quinone ($\mu\text{g.}$)	α -Toco- pherol quinone ($\mu\text{g.}$)	Ubi- quinone-9 ($\mu\text{g.}$)
3	Light	0.262	2.5	0.7	14.8	28	8	8
	Dark	0.350	3.5	0	0	5	0	4
5	Light	0.537	8.5	4.4	66.4	169	34	25
	Dark	0.725	13.5	0	4.8	87	0	22
Dark-grown shoots excised and exposed to light* for:								
	3 hr.		13.5	Trace	3.5	59	0	20
	48 hr.		13.5	1.83	83	180	20	32
	Dark-grown shoots exposed to light for 48 hr.	0.899	14	4.56	122	203	43	38
7	Light	0.694	11	5.1	133	258	60	38
	Dark	0.788	15	0	6.4	92	0	30

* See Fig. 3 for intermediate values.

Fig. 2. Effect of white light on the formation of chlorophyll, β -carotene, plastoquinone, α -tocopherolquinone and ubiquinone by excised etiolated maize shoots. $\circ$, Chlorophyll; Δ , plastoquinone; $\square$, ubiquinone-9; $\bullet$, α -tocopherolquinone; $\blacktriangle$, β -carotene.

was greatest in maize, i.e. a fourfold increase, compared with a twofold increase in barley, but this is only to be expected since the difference in plastoquinone concentrations in etiolated compared with normal green shoots of barley is much less than in maize (Tables 2, 4 and 5).

Effect of light on phylloquinone, α -tocopherolquinone and α -tocopherol synthesis by excised etiolated maize shoots. The above experiment had shown that on illumination of etiolated shoots there was a marked synthesis of α -tocopherolquinone. Since qualitative observations suggested that this compound was not formed at the immediate expense of α -tocopherol, there appeared to be too little α -tocopherol to account for all the α -tocopherolquinone formed, and it was decided to determine the exact stoichiometric relationship. The effect of light on the phylloquinone concentration was also investigated, since earlier studies on the concentration of this compound in etiolated and green shoots had not been clear-cut.

It was found that, although illumination for 24 hr. of 6-day-old excised dark-grown maize shoots led to an increase in α -tocopherolquinone content from 'trace' to $287 \mu\text{g./100}$ shoots, this treatment had little effect on α -tocopherol concentrations, which were 209 and $227 \mu\text{g./100}$ shoots at zero time and 24 hr. respectively. The phylloquinone concentration during the experimental period rose from 90 to $140 \mu\text{g./100}$ shoots.

Since the α -tocopherol concentration is unchanged at the end of the experimental period it appeared that no α -tocopherolquinone was being formed at the immediate expense of α -tocopherol. A more complete time study has demonstrated that even during the early stages of α -tocopherolquinone synthesis α -tocopherol concentrations are unaffected (Griffiths, 1965). However, the possibility exists that any α -tocopherol or γ -tocopherol (through α -tocopherol) converted into α -tocopherolquinone is rapidly replenished to maintain a constant pool size.

Fig. 3. Effect of white light on the formation of chlorophyll, β -carotene, plastoquinone, α -tocopherolquinone and ubiquinone by excised etiolated barley shoots. $\circ$, Chlorophyll; Δ , plastoquinone; $\square$, ubiquinone-9; $\bullet$, α -tocopherolquinone; $\blacktriangle$, β -carotene.

Thus under these experimental conditions phyloquinone is actively synthesized. After excision of the shoots, if the phyloquinone concentration first falls in a similar manner to β -carotene and plastoquinone concentrations (see above), then appreciable synthesis of this component occurs.

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REFERENCES

- Arnon, D. I. (1949). *Plant Physiol.* **24**, 41.
 Avigan, J., Goodman, D. S. & Steinberg, D. (1963). *J. Lipid Res.* **4**, 100.
 Bailey, J. L. & Whyborn, A. G. (1963). *Biochim. biophys. Acta*, **78**, 163.
 Crane, F. L. (1959). *Plant Physiol.* **34**, 128.
 Crane, F. L. (1961). In *Ciba Found. Symp.: Quinones in Electron Transport*, p. 36. Ed. by Wolstenholme, G. E. & O'Connor, C. M. London: J. and A. Churchill Ltd.
 Crane, F. L. (1965). In *Biochemistry of Quinones*, p. 183. Ed. by Morton, R. A. New York and London: Academic Press Inc.
 Dam, H. & Glavind, J. (1938). *Biochem. J.* **32**, 485.
 Dam, H., Glavind, J. & Nielson, R. (1940). *Hoppe-Seyl. Z.* **265**, 80.
 Das, B. C., Lounasmaa, M., Tendile, C. & Lederer, E. (1965). *Biochem. biophys. Res. Commun.* **21**, 318.
 Egger, K. (1963). *Ber. dtsh. bot. Ges.* **76**, 370.
 Goodwin, T. W. (1958). *Biochem. J.* **70**, 612.
 Green, J. (1958). *J. Sci. Fd Agric.* **9**, 801.
 Griffiths, W. T. (1965). Ph.D. Thesis: University College of Wales, Aberystwyth.
 Griffiths, W. T., Threlfall, D. R. & Goodwin, T. W. (1964). *Biochem. J.* **90**, 40P.
 Hemming, F. W., Morton, R. A. & Pennock, J. F. (1963). *Proc. Roy. Soc. B*, **158**, 291.

- Henninger, M. & Crane, F. L. (1963). *Biochemistry*, **2**, 1168.
- Holm-Hansen, O., Pon, N. C., Nishida, K., Moses, V. & Calvin, M. (1959). *Physiol. Plant.* **12**, 475.
- Kay, R. E. & Phinney, B. (1956). *Plant Physiol.* **31**, 226.
- Kegel, L. P., Henninger, M. & Crane, F. L. (1962). *Biochem. biophys. Res. Commun.* **8**, 294.
- Kofler, M., Langemann, A., Ruegg, R., Chopard-dit-Jean, L. H., Raymond, O. & Isler, O. (1959). *Helv. chim. Acta*, **42**, 1283.
- Lichtenthaler, H. K. & Calvin, M. (1964). *Biochim. biophys. Acta*, **79**, 30.
- Mercer, E. I., Davies, B. H. & Goodwin, T. W. (1963). *Biochem. J.* **87**, 317.
- Mercer, E. I. & Goodwin, T. W. (1962). *Biochem. J.* **85**, 13r.
- Page, A. C., jun., Gale, P. H., Koniuszy, F. R. & Folkers, K. (1959). *Arch. Biochem. Biophys.* **85**, 474.
- Pennock, J. F., Neiss, G. & Mahler, H. R. (1962). *Biochem. J.* **85**, 530.
- Redfearn, E. R. (1965). In *Biochemistry of Quinones*, p. 149. Ed. by Morton, R. A. New York and London: Academic Press Inc.
- Rowland, R. L., Latimer, P. H. & Giles, J. A. (1965a). *J. Amer. chem. Soc.* **78**, 4680.
- Rowland, R. L., Latimer, P. H. & Giles, J. A. (1965b). *J. Amer. chem. Soc.* **78**, 4680.
- Stevenson, J., Hemming, F. W. & Morton, R. A. (1964). *Biochem. J.* **88**, 52.
- Stumpf, P. K. & James, A. T. (1963). *Biochim. biophys. Acta*, **70**, 20.
- Threlfall, D. R. & Goodwin, T. W. (1963). *Biochim. biophys. Acta*, **78**, 532.
- Threlfall, D. R. & Goodwin, T. W. (1964). *Biochem. J.* **90**, 40r.
- Threlfall, D. R. & Goodwin, T. W. (1967). *Biochem. J.* **103**, 573.
- Threlfall, D. R. & Griffiths, W. T. (1966). *Biochemistry of Chloroplasts*, vol. 2, p. 254. New York and London: Academic Press Inc.
- Threlfall, D. R., Griffiths, W. T. & Goodwin, T. W. (1965). *Biochim. biophys. Acta*, **102**, 614.
- Threlfall, D. R., Griffiths, W. T. & Goodwin, T. W. (1967). *Biochem. J.* **103**, (in the Press).