Nature, Intracellular Distribution and Formation of Terpenoid Quinones in *Euglena gracilis*

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1 Light-grown cells of *Euglena gracilis* strains Z, var. *bacillaris* and 1224/5g contain phyloquinone, plastoquinone, α -tocopherol, α -tocopherolquinone and ubiquinone-9 (i.e. ubiquinone with 9 isoprene units/mol.). 2. The concentration (per g. dry wt.) of plastoquinone (and chlorophyll) in light-grown cells of strain Z was governed by the composition of the culture medium and age of the cells. Highest yields of plastoquinone were obtained under autotrophic conditions, the concentration reaching a maximum after 6–8 days' growth. The concentrations were less in heterotrophic media. The concentration of ubiquinone was relatively unaffected by the age of the cells or composition of the medium. 3. In light-grown cells of strain Z plastoquinone, α -tocopherolquinone and α -tocopherol were mainly localized in the chloroplast; ubiquinone was found to be in the mitochondria. 4. Etiolated (dark-grown) cells of strain Z contained no phyloquinone, plastoquinone or α -tocopherolquinone; α -tocopherol was present in lower concentrations compared with light-grown cells; ubiquinone concentrations were similar to those for light-grown cells. The presence of α -tocopherol in etiolated cells suggested that this chromanol was not entirely confined to the chloroplast. 5. On illumination of etiolated cells of strain Z the chloroplastidic components plastoquinone, α -tocopherolquinone and α -tocopherol were synthesized in step with chloroplast formation. Ubiquinone concentrations, as expected, were unaffected. 6. [2-¹⁴C]Mevalonic acid, the specific distal terpenoid precursor, was not incorporated into any of the terpenoid components examined. This was attributed to the impermeability of the cell wall to this compound, rather than to a novel pathway of terpenoid biosynthesis.

It is now generally appreciated that one or more quinones possessing terpenoid side chains are present in any of the structures within the cell carrying out electron-transport reactions, e.g. mitochondria, chloroplasts and chromatophores. Indeed, an impressive body of evidence has accumulated that implicates these compounds in the electron-transport and phosphorylation reactions associated with such particles (see, e.g., Brodie, 1965; Green & Brierley, 1965; Arnon & Crane, 1965).

The present series of investigations were undertaken with the aim of studying the synthesis in photosynthetic plant tissues of the terpenoid quinones, ubiquinone, plastoquinone and phyloquinone (vitamin K₁). We were concerned, first, with the formation of these compounds in terms of chloroplast development, and, secondly, with the biosynthesis of the prenyl portions of the quinone molecules, both in relationship to each other and to

the other isoprenoid compounds, in particular β -carotene and the phytosterols, occurring in such tissues. However, during these studies the isolation of further quinones from plant tissues, more particularly the chloroplast, led to the scope of the investigations being widened to include these new compounds, and in some cases related non-quinoid compounds, such as tocopherols.

At the inception of these studies the terpenoid quinones recognized as occurring in green plant tissues were ubiquinone, plastoquinone and phyloquinone. It had been shown that most phyla of the green plant kingdom contain either ubiquinone-10, ubiquinone-9 or ubiquinone-8 (the numerals refer to the numbers of isoprene units/mol.), the higher homologues being found in higher plants (Lester & Crane, 1959; Page, Gale, Koniuszy & Folkers, 1959). Intracellular-distribution studies confirmed that in plant tissues, as in animal tissues (Hemming, Pennock & Morton, 1958), ubiquinone was associated with the mitochondria (Crane, 1959a;

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see also Crane, 1965). Plastoquinone has been isolated from all green oxygen-producing photosynthetic tissues examined, where it is mainly if not entirely confined to the chloroplast (see Redfearn, 1965). Although phyloquinone had received considerable attention there was some doubt, owing mainly to the difficulty attached to its isolation and subsequent estimation, about its occurrence in all photosynthetic plant tissues. It now appears that it occurs in the photosynthetic tissues of all higher plants, and that it is located mainly in the chloroplast (Lester & Crane, 1959; Kegel & Crane, 1962; Lichtenthaler & Calvin, 1964). Plastoquinone and phyloquinone are not wholly confined to photosynthetic tissues. Thus plastoquinone occurs, at concentrations much below those found in photosynthetic tissues, in maize roots (Lester & Crane, 1959), cauliflower buds (Lester & Crane, 1959), etiolated maize and barley shoots (Griffiths, Threlfall & Goodwin, 1967) and cambial-tissue cultures of Paul's Scarlet Rose (Threlfall & Goodwin, 1963). Similarly, phyloquinone is present in dark-grown seedlings of *Picea canadensis* (Dam & Glavind, 1938; Dam, Glavind & Nielson, 1940). Crane (1961) suggested that the occurrence of plastoquinone in non-photosynthetic tissues may be indicative of the potential for chloroplast development in such tissues. This suggestion has been amplified by Threlfall & Griffiths (1966).

In addition to phyloquinone and plastoquinone no fewer than nine quinones have now been isolated from chloroplasts (mainly spinach chloroplasts). These are demethyl-vitamin K₁ (McKenna, Henninger & Crane, 1964), the plastoquinone homologues plastoquinone-4 isolated from horse-chestnut leaves (Eck & Trebst, 1963) and plastoquinone-3 isolated from spinach chloroplasts (Misiti, Moore & Folkers, 1965), the plastoquinone analogues plastoquinone B (Kegel, Henninger & Crane, 1962), plastoquinone C and plastoquinone D (Henninger & Crane, 1963), and α -tocopherolquinone, β -tocopherolquinone and γ -tocopherolquinone (Henninger, Dilley & Crane, 1963). Demethyl-vitamin K₁, β -tocopherolquinone and γ -tocopherolquinone occur in extremely small amounts. The structures of these compounds, with the exception of plastoquinone B, plastoquinone C and plastoquinone D, are well established. Characterization studies suggest that plastoquinone C and plastoquinone D are monohydroxy derivatives of plastoquinone, the hydroxyl group being located in the second isoprene unit from the quinone ring (Threlfall, Griffiths & Goodwin, 1965; Das, Lounasmaa, Tendile & Lederer, 1965; Threlfall & Griffiths, 1966). Plastoquinone B appears to be a more unsaturated form of plastoquinone, the unsaturation occurring in the second isoprene unit from the ring (Das *et al.* 1965).

The isolation of α -tocopherolquinone from the chloroplast necessarily meant that any study of this compound should include the chromanol α -tocopherol (and related compounds), from which α -tocopherolquinone can be formed by a suitable oxidation step. In plant tissues all members of the tocopherol and tocotrienol series have been found (see Whittle, Dunphy & Pennock, 1966); although tocopherols other than α -tocopherol are generally absent from photosynthetic tissues, they frequently occur in seeds and fruits (Green, 1963). In some higher plants γ -tocopherol [maize (Green, 1958), *Taxus baccata* and *Hedera helix* (Booth, 1963)] and δ -tocopherol (*T. baccata* and *H. helix*; Booth, 1963) may be present, in addition to α -tocopherol. Intracellular-distribution studies have shown that in these tissues α -tocopherol is present in the chloroplast, whereas γ -tocopherol and δ -tocopherol are found outside the chloroplast (Booth, 1963).

Studies by Griffiths (1965) and Bucke, Leech, Hallaway & Morton (1966) have shown that the photosynthetic apparatus of oxygen-producing organisms invariably contains phyloquinone, plastoquinone, α -tocopherolquinone and α -tocopherol, plastoquinone B, plastoquinone C and plastoquinone D being absent from many tissues. This prompted Threlfall *et al.* (1965) to suggest that their distribution pattern may be of phylogenetic value. It also appears that, with the exception of α -tocopherolquinone and α -tocopherol, all these quinones are unique to oxygen-producing photosynthetic organisms.

Fuller, Smillie, Rigopoulos & Yount (1961) had previously stated that autotrophically grown *Euglena gracilis* contained plastoquinone (1.49 μ moles/g. dry wt.) and ubiquinone-8 (0.21 μ mole/g. dry wt.). Apoplastidic (dark-grown) organisms contained no plastoquinone and slightly higher concentrations of ubiquinone-8 (0.29 μ mole/g. dry wt.). Green, Price & Gare (1959) had also shown that autotrophic cultures of *E. gracilis* contained α -tocopherol (155 μ g./g. fresh wt.), no other forms being detected.

We now report on the nature, intracellular distribution and formation of the terpenoid quinones and related compounds by *E. gracilis* strain Z. The results of these investigations have been communicated briefly (Threlfall & Goodwin, 1964).

EXPERIMENTAL

Biological methods

Organisms. *E. gracilis* strain Z and *E. gracilis* var. *bacillaris* were obtained from the Culture Collection of Algae and Protozoa, Cambridge. *E. gracilis* strain 1224/5g was a gift from Dr J. T. O. Kirk.

Culture. (a) Maintenance. The various strains of *E. gracilis* were maintained on half-strength Oxoid nutrient

agar (CM4) slopes in subdued light at room temperature; transfers were made monthly. The organisms, for experimental purposes, were maintained by weekly transfers through autotrophic liquid culture. Periodic microscopic examinations of the cultures were made to check for possible contaminants. Fresh liquid subcultures were prepared from slopes whenever the organisms showed any signs of discoloration or clumping.

(b) Cultural conditions. The organisms were grown on a variety of media and under various cultural conditions in these studies. The medium used for most experiments was the autotrophic medium of Pringsheim & Pringsheim (1952), consisting of 0.5% proteose peptone (Oxoid) and 0.2% yeast extract (Difco) in tap water. Other media employed were the heterotrophic media of Pringsheim & Pringsheim (1952), containing sodium acetate, and of Wolken (1961), rich in glucose and organic acids.

The medium was dispensed in 100 ml. or 750 ml. volumes in 250 ml. Erlenmeyer or 2 l. long-necked flasks respectively. Experimental cultures were, unless otherwise stated, inoculated from an actively growing 5–6-day-old autotrophic liquid culture. In balance experiments 5 ml. of culture was added/100 ml. of medium.

Green cultures were grown statically on a light-table kept at 28°. Continuous illumination was provided by a fluorescent strip-light (Metrovic Daylight 80w) 6–8 in. from the cultures. Etiolated (dark-grown) cultures were grown in flasks wrapped in aluminium foil and kept in the dark. All cultures were shaken vigorously for 30 sec. twice a day.

Harvesting and determination of dry weight. The cells were harvested, by low-speed centrifugation in a Martin-Christ bench centrifuge at speed 9, into a weighed centrifuge tube. The harvested material was washed twice by resuspension in distilled water followed by centrifugation. The centrifuge tube with the harvested material was then reweighed and, after stirring the wet cell mass thoroughly with a glass rod, a sample was taken for freeze-drying. From these values the total dry weight of the sample was calculated.

Analytical methods

Cell disruption and fractionation. A 2 l. volume of culture was harvested by centrifugation and, after washing, the cells were collected in a weighed 50 ml. glass MSE centrifuge tube. Samples were then taken of the wet cell mass for quinone and dry-weight determination (100 and 20 mg. respectively). The remaining cells were suspended in 12 ml. of ice-cold 10% (w/v) sucrose solution and treated ultrasonically, with the Mullard Ultrasonicator with a $\frac{1}{4}$ in. probe, for periods of 90, 40 and 20 sec. with 1 min. intervals between 'bursts'. Throughout this treatment the tube containing the preparation was surrounded by crushed ice. Immediately on completion of the ultrasonic treatment 15 ml. of ice-cold 10% (w/v) sucrose solution was added to the preparation to give a final volume of 30 ml. of suspension with a concentration of 10% (w/v) sucrose. The disruption was followed by microscopic inspection and it was apparent that even after the third exposure to ultrasonic vibrations, although a large number of cells were still intact (about 80%), chloroplasts and cell walls were present in the medium. Further, the disrupted material showed no tendency to clump.

The preparation of whole and broken cells was centrifuged for 3 min. at 140g (MSE bench centrifuge); the pellet (whole cells, large particles and trapped chloroplasts) was

resuspended in 20 ml. of 10% (w/v) sucrose and subjected to the same centrifugation. The supernatants were combined and centrifuged once more at 140g for 5 min. to remove any residual unbroken cells. Before subjecting the supernatant to a scheme of differential centrifugation a sample (one-fifth of the volume) was taken for chlorophyll and quinone determination. The first fraction collected, sedimenting at 3000g for 5 min. (Servall Superspeed model RC-2 centrifuge with SS-34 head), was labelled 'chloroplasts', although cell walls, nuclear material and paramylum grains were also present. 'Mitochondria' and 'microsomes' were sedimented by centrifugation at 20000g for 15 min. (Servall Superspeed model RC-2 centrifuge with SS-34 head) and 105000g for 60 min. (Beckmann Spinco model L preparative centrifuge) respectively. All the pellets were green. The final supernatant was a pale-yellowish colour and had orange curds floating on the surface; these possibly had arisen from the eye spots.

Extraction of the lipid. (a) Whole cells. Although in this work a variety of extraction procedures were tried (see the Results and Discussion section), the routine method used was a total lipid extraction of the freshly harvested biological material. The cell mass, in the Martin-Christ centrifuge tube, was extracted once with boiling ethanol followed by three extractions with hot ethanol-ether (1:3, v/v); the cells from 100 ml. of medium require 20 ml. of ethanol and 100 ml. (i.e. 3×33 ml.) of ethanol-ether mixture. The cell debris was separated from the extracts by centrifugation and the supernatants were decanted into a 250 ml. separating funnel. At this stage the cells were usually colourless, although with old cultures (about 9 days) it is difficult to remove the last traces of chlorophyll.

The combined extracts were swirled gently to mix the ether and ethanol phases and then washed free of ethanol by carefully running water down the sides of the funnel; if care is taken no colour is taken out by the ethanol-water phase and the need to re-extract this washing does not arise (if any colour was present a back-extraction was performed). The washings were continued until all traces of ethanol were removed. The ethereal layer containing all the colour was dried over anhydrous Na_2SO_4 (A.R.) for 1 hr. and filtered. The extract was then made up to a known volume, a sample taken for a chlorophyll determination (see below) and the remainder evaporated to dryness under a stream of N_2 .

(b) Intracellular fractions. The particulate cell fractions were extracted in a manner identical with the procedure outlined above, after making suitable reductions in the volumes of solvents used. Chlorophyll was determined in the dry-ether extracts. The supernatant fraction was treated with hot ethanol (4 vol.), diluted with ice-cold water (10 vol.) and extracted four times with ether. The combined extracts were treated as just described.

Chromatography. (a) Column chromatography. The preliminary fractionation of the total lipid (or unsaponifiable) extract was carried out on a column of Brockmann grade III acid-washed alumina (Woelm anionotropic). Various workers (see, e.g., Crane, 1961) have commented on the lability of quinones towards alumina; however, in recovery experiments with plastoquinone, ubiquinone, α -tocopherolquinone and phyloquinone we have obtained quantitative recoveries on Brockmann grade III alumina columns. The ratio of lipid to adsorbent is critical and we employ 5, 10, 15 or 20 g. of alumina/0–75, 75–125, 125–175 or 175–225 mg. of lipid; the ratio of diameter to length of

Table 1. *Elution sequence off acid-washed alumina columns of ultraviolet- and visible-light-absorbing terpenoids found in E. gracilis*

The adsorbent was Brockmann grade III acid-washed alumina (Woelm), and elution was with light petroleum (b.p. 40–60°) containing increasing amounts of ether. Experimental conditions are given in the text. The compounds listed are not always spectroscopically detectable in the crude fractions. This is always the case with phyloquinone and usually the case with α -tocopherol.

Eluent	Compounds eluted
0% E/P	β -Carotene, phyloquinone*
1% E/P	Plastoquinone, $\Delta^{5,7}$ -sterol ester
3% E/P	α -Tocopherol
5% E/P	Ubiquinone, trace of α -tocopherol
8% E/P	—
12% E/P	$\Delta^{5,7}$ -Sterol
20% E/P	α -Tocopherolquinone, $\Delta^{5,7}$ -sterol

* Phyloquinone is not always eluted entirely in this fraction; however, Griffiths (1965) has shown that the addition of 0.25% ether results in the elution of all this compound in the first fraction.

column is kept between 1:5 and 1:8. The alumina is weighed, covered with light petroleum (b.p. 40–60°) and the requisite amount of water added for deactivation; after dispersion of the water globules by gentle agitation, the adsorbent is washed with light petroleum into a suitable column plugged with non-absorbent cotton wool.

The lipid is taken up in the minimum volume of light petroleum (b.p. 40–60°), with slight warming if necessary, and applied carefully to the column. The column is then developed by stepwise elution with 0%, 1%, 3%, 5%, 8%, 12% and 20% E/P,* 10 ml. of each fraction being run/g. of adsorbent.

The fractions from the column are evaporated to dryness (under N_2) in tared vessels, desiccated to remove the last traces of solvent, weighed and the spectra determined in a suitable solvent. Table 1 lists the elution pattern of some u.v.- and visible-light-absorbing lipids found in *E. gracilis*. In balance experiments the amounts of β -carotene, plastoquinone, ubiquinone, α -tocopherolquinone and α -tocopherol were estimated at this stage (see below). Phyloquinone requires further purification before it can be assayed. Some of the fractions eluted from the preliminary alumina column were purified further by rechromatography on the same sized columns of Brockmann grade II acid-washed alumina as originally used.

(b) Thin-layer chromatography. The quinones and chromanols were identified and in some cases resolved and purified by thin-layer chromatography. The adsorbent used throughout was Kieselgel G (according to Stahl) for thin-layer chromatography obtained from E. Merck A.-G., Darmstadt, Germany. The plates used were 250 μ thick and after preparation were stored *in vacuo*.

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

Although the quinones can be resolved into their respective classes by adsorptive thin-layer chromatography, the various homologues cannot be separated from each other, e.g. ubiquinone-10, ubiquinone-9 and ubiquinone-8 all run with the same R_F . To identify the homologue it is necessary to subject the sample to reversed-phase partition chromatography.

Reversed-phase partition chromatography on thin layers was carried out on plates impregnated with a 5% (v/v) solution of medicinal paraffin in light petroleum (b.p. 60–80°) (Threlfall & Goodwin, 1963; Goodwin, 1964). The solvent system is given in Table 2.

The samples along with standards were applied as discrete spots from capillary tubes. The plates were then developed in a saturation chamber (Davies, 1963). In the early part of the work the compounds were detected by spraying with suitable reagents. Quinones were located with leucomethylene blue (Linn *et al.* 1959) or phosphomolybdic acid (Bolliger, 1962); chromanols and quinols were revealed with the Emmerie–Engel reagent (Pennock, Neiss & Mahler, 1962). Later, Rhodamine 6G-impregnated plates were used. This allowed the detection of the quinone and chromanol spots under u.v. light (Threlfall *et al.* 1965). Identification was not based on R_F values, but rather by chromatography both with and against authentic samples. Table 2 lists some typical R_F values obtained for the various systems used.

For preparative work the sample was dissolved in a minimum volume of cyclohexane (10–20 μ l. or 0.1–0.2 ml.) in a combustion tube and applied as a 5–15 cm. strip from a 10 μ l. or 0.1 ml. pipette; markers were then spotted at each end of the band.

After development of the plate the spots were located and quickly scraped off into ether; the supernatant and washings were combined and separated from the adsorbent with a Pregl filter stick.

(c) Paper chromatography. Reversed-phase paper chromatography was used as an additional aid for identifying the quinones. The method employed was that described by Lawson, Threlfall, Glover & Morton (1961) with the substitution of medicinal paraffin for petroleum jelly. This shortens the running time from 12 hr. to 3 hr. The quinones were located with a Hanovia Chromatolite lamp; plastoquinone, ubiquinone and α -tocopherolquinone appear as dark-blue spots and vitamin K₁ as a strongly fluorescing green zone. The quinones were also located by spraying with leucomethylene blue (see above).

Quantitative estimation of components. (a) Chlorophyll. Chlorophyll was determined as chlorophyll ($a+b$) by the method of Arnon (1949).

(b) β -Carotene. This pigment was eluted in the light-petroleum fraction on column chromatography of the lipid extract (Table 1). It was estimated spectroscopically by using $E_{1\text{cm}}^{1\%}$ 2500 at λ_{max} 451 m μ (in light petroleum) (Goodwin, 1955).

(c) Plastoquinone, ubiquinone and α -tocopherolquinone. The quantitative estimations of plastoquinone, ubiquinone and α -tocopherolquinone were carried out directly on the appropriate fractions obtained from chromatography of the lipid extract (Table 1).

The fraction was dissolved in ethanol and the u.v. spectrum determined. The quinone was then converted into the quinol by the addition of a small crystal of NaBH₄ to the spectrophotometer cell, and after waiting 30 sec. the reduced spectrum was determined. The amount of quinone

Table 2. *Chromatographic systems used for the final purification and identification of terpenoid quinones and related compounds*

Thin-layer chromatograms were developed in a saturation chamber (Davies, 1963); compounds were never identified on the basis of R_F values alone; appropriate marker spots were always run. Liquid paraffin was the stationary phase in both reversed-phase chromatographic systems. Experimental details are given in the text.

Compound	R_F values on Kieselgel G plates					R_F values on paper	
	Benzene-light petroleum (2:3, v/v)	Benzene-chloroform (1:1, v/v)	Benzene	Ethyl acetate-benzene (3:22, v/v)	Water-acetone (1:19, v/v) (reversed phase)	Water- <i>NN</i> -dimethylformamide (1:39, v/v) (reversed phase)	
Phylloquinone	0.39			0.98	0.34		0.42
Plastoquinone	0.29			0.98	0.20	0.14	0.1
Ubiquinone-10					0.34		0.26
Ubiquinone-9			0.30		0.45		0.41
Ubiquinone-8					0.52		0.56
α -Tocopherol	0.18	0.47*	0.54				
α -Tocopherolquinone	0.00			0.48	0.68	0.82	

* For the R_F values of other tocopherols in this system see Pennock *et al.* (1962).

Table 3. *Factors used for the spectroscopic determination of quinones by reduction with sodium borohydride*

All determinations were carried out in spectroscopically pure ethanol. Experimental conditions are given in the text.

Quinone	$\lambda_{\max}$ (m μ)	$\Delta E_{1\text{cm}}^{1\%}$	$\epsilon_{\text{ox}} - \epsilon_{\text{red}}$	Reference
Plastoquinone	255	198	14800*	Crane (1959b)
Ubiquinone-9	275	154	12250	Lawson <i>et al.</i> (1961)
Ubiquinone-10	275	142	12250	
α -Tocopherolquinone	262	397	17700*	Dilley & Crane (1963)

* Calculated from $\Delta E_{1\text{cm}}^{1\%}$ value.

present was calculated by measuring the fall in extinction at the wavelengths of maximum absorption. Table 3 lists the factors used.

(d) α -Tocopherol. Although tocopherols have characteristic u.v. spectra, it is not normally practicable to use these characteristics for their assay since the curves are easily masked by interfering substances. This occurs more so in plant than in animal tissues.

Equivalent samples were taken from the 3%- and 5%-E/P fractions from the preliminary chromatography and the amount of tocopherol (as α -tocopherol) was estimated by using the modified Emmerie-Engel reaction described by Pennock *et al.* (1962).

The possibility of erroneously high readings caused by the presence of interfering compounds in the fractions was checked by: (i) quantitative thin-layer chromatography of a sample followed by spectroscopic assay of the recovered chromatol (Pennock *et al.* 1962); (ii) oxidation of a further sample to convert any α -tocopherol into α -tocopherolquinone (Dilley & Crane, 1963) followed by chromatography

on alumina to remove any ubiquinone, and estimation of the recovered α -tocopherolquinone by using NaBH_4 (see above). In both cases the values recorded agreed well with the values obtained with the Emmerie-Engel reagent.

(e) Phylloquinone. This naphthaquinone was estimated from its absorption at $\lambda_{\max}$ 249 m μ in cyclohexane by using $E_{1\text{cm}}^{1\%}$ 419.

Spectroscopy. The u.v. and visible spectra were determined with the Perkin-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 redistilled over reduced iron immediately before use.

Ethanol and cyclohexane were of spectroscopic grade.

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Table 4. *Efficiency of various methods of lipid extraction for the isolation of plastoquinone and ubiquinone*

A sample (30g. wet wt., 7.2g. dry wt.) of 6-day-old heterotrophic light-grown cells was divided into four equal portions and extracted by the procedures listed below. The extracts were then examined for plastoquinone and ubiquinone by our routine procedures.

Extraction procedure	Lipid (mg./g. dry wt.)	Recovery (μ moles/g. dry wt.)		
		Chlorophyll	Plastoquinone	Ubiquinone-9
(1) Total lipid*	267	10.7	0.21	0.13
(2) Freeze-dried†	221	6.4	0.06	0.10
(3) Acetone/sand‡	215	7.3	0.10	0.02
(4) Saponification§	91	10.7	0.20	0.004

* Routine procedure described in the text.

† After Fuller *et al.* (1961).

‡ Wet cells were ground with sand under acetone, and the extracts obtained were treated as for 'total lipid'.

§ After Hemming, Morton & Pennock (1963); the method involves the use of pyrogallol.

RESULTS AND DISCUSSION

Isolation of quinones (plastoquinone and ubiquinone) from E. gracilis. A number of different methods of extracting the cells for quinones were examined. *E. gracilis* strain Z was grown in the light on a medium rich in glucose and organic acids (Wolken, 1961) for 6 days, by which time appreciable growth had taken place; the cells were then harvested, split into four equal portions and extracted by a variety of methods (Table 4). The lipid extracts were chromatographed on Brockmann grade III alumina columns; 0%, 1%, 3% and 5% E/P fractions were collected and the 1% and 5% E/P fractions examined spectroscopically for the presence of plastoquinone and ubiquinone respectively. The 1% E/P fraction from the lipid extract exhibited absorption bands at $\lambda_{\max}$ 262, 282 and 295 m μ and a shoulder at 255 m μ (Fig. 1), whereas that from the saponified extract had a spectrum identical with that of plastoquinone ($\lambda_{\max}$ 255 m μ). Rechromatography of the 1% E/P lipid fraction on a Brockmann grade II column separated plastoquinone (2% E/P) from the second component (4% E/P; $\lambda_{\max}$ 272, 282 and 294 m μ). This was shown to be a $\Delta^5,7$ -sterol ester. The presence of plastoquinone was further established by plotting the difference spectrum after reduction with sodium borohydride; it was identical with that of authentic plastoquinone.

The sodium borohydride method for quantitative determination of plastoquinone could be carried out on fractions that also contained the $\Delta^5,7$ -sterol because the reagent had no effect on the spectrum of the sterol, and in trial experiments known mixtures of plastoquinone and the sterol were accurately assayed for plastoquinone.

The ubiquinone-containing fractions all had peaks at $\lambda_{\max}$ 275 m μ (in ethanol). Reduction with

Fig. 1. Ultraviolet spectra in ethanol (15 ml.) of plastoquinone-containing fraction (1% E/P) obtained from chromatography of *Euglena* lipid (from heterotrophic cells) on alumina. —, 1% E/P fraction; —*R*—, after treatment with NaBH₄.

sodium borohydride and plots of the difference spectra gave curves similar to authentic ubiquinone difference spectra.

On reversed-phase paper chromatography the plastoquinone-containing fractions all ran as authentic plastoquinone-9, in agreement with the observations of Fuller *et al.* (1961). The presence of spots showing strong green fluorescence under u.v. light and running with similar *R_F* to phyloquinone was also noted. The ubiquinone-containing fractions, although streaking badly, ran as ubiquinone-9 and not as the ubiquinone-8 form reported by Fuller *et al.* (1961).

The quantitative results for the experiments (Table 4) showed that the highest recovery of quinones was obtained by using the 'ethanol-ether' procedure, i.e. the routine method; extraction of freeze-dried cells and the saponification procedures both gave reasonable recoveries; grinding with sand under acetone gave a very poor yield. It was later demonstrated that, although the recoveries of plastoquinone from heterotrophic cells after saponification were quite good, saponification of autotrophic cells (which were used for most of the experimental work) resulted in a drastic loss of plastoquinone. The technique adopted for all this work was the 'ethanol-ether' procedure, since the extraction was rapid and complete.

Effects of media and time on the plastoquinone and ubiquinone concentrations of E. gracilis. The most striking results of the experiment just described are that the plastoquinone concentrations (Table 4) were some sevenfold lower than the value (1.49 μ moles/g. dry wt.) quoted by Fuller *et al.* (1961). Ubiquinone values, on the other hand, were in better agreement. It was considered that the low plastoquinone concentrations may have been due to the fact that Fuller *et al.* (1961) had used autotrophically grown cells. To explore this possibility the organisms were cultured on a variety of media under identical conditions of light and temperature, and the dry weight, chlorophyll, plastoquinone and ubiquinone were determined. The effect of time on these factors was also studied (Table 5 and Fig. 2).

In heterotrophic light-grown cells of strain Z it was found that, although growth was considerably better, the concentrations of plastoquinone and chlorophyll were significantly lower than the

corresponding concentrations in autotrophically grown cells of a similar age. The fall in concentration increased with increasing complexity of medium. Also, until the cells were 6 days old the concentrations of chlorophyll and plastoquinone per unit volume of medium were significantly lower. After 6 days the concentrations of chlorophyll and plastoquinone per unit volume and per g. dry wt. in heterotrophic cultures showed an increase until at 12 days old the concentration of chlorophyll and plastoquinone per unit volume for both heterotrophic media and the concentration per g. dry wt. for the weakly heterotrophic medium (0.1% sodium acetate) were similar to the values recorded for 6- and 12-day-old autotrophic cultures. The concentrations of chlorophyll and plastoquinone in the cells from the richly heterotrophic cultures never reached the concentrations found in autotrophically grown cells. In all cultures ubiquinone values showed little variation. Smillie (1963) has shown that growing *Euglena* cells in heterotrophic media suppresses the development of chloroplasts and this leads us to suggest that the lower concentrations of chlorophyll and plastoquinone observed in heterotrophic cultures are a reflexion of this suppression. However, in accepting this, consideration must be given to the fact that our method of expression of results (per g. dry wt.) makes no allowance for differences in cell size or mass. Thus, although the trends are undoubtedly correct, the actual differences may be exaggerated.

The kinetic study of the synthesis of chlorophyll, plastoquinone and ubiquinone by autotrophic cultures showed that ubiquinone concentrations were relatively unaffected but that chlorophyll and

Table 5. *Effects of media and time on plastoquinone and ubiquinone concentrations of E. gracilis strain Z*

Cells were cultured in the light on a variety of media, and the chlorophyll, plastoquinone and ubiquinone contents determined after various periods of growth. Two flasks (each containing 100 ml. of medium) were taken for the analysis of all cultures up to 4 days old; after this only one flask was taken. Fig. 2 records the whole time-course of chlorophyll, plastoquinone and ubiquinone formation by autotrophically grown cells.

Medium	Time of growth (days)	Dry wt. (mg./flask)	Concn./flask (100 ml. of medium)				Concn./g. dry wt.		
			Lipid (mg.)	Chlorophyll (mg.)	Plastoquinone (μ g.)	Ubiquinone (μ g.)	Chlorophyll (mg.)	Plastoquinone (μ g.)	Ubiquinone (μ g.)
Autotrophic	4	74.6	17.8	1.54	66.9	15.6	20.6	897	209
	6	108	24.6	3.75	230	21.4	34.7	2120	198
	12	122	34.1	5.92	272	26.0	48.5	2221	213
Heterotrophic									
(a) 0.1% sodium acetate	4	56.4	9.7	0.56	22.4	9.3	10.0	397	164
(Pringsheim & Pringsheim, 1952)	6	126	24.2	2.27	134	24.4	18.0	1060	194
	12	168	39.3	7.65	309	26.9	45.6	1840	160
(b) 1% glucose and organic acids (Wolken, 1961)	4	110	16.3	0.83	18.8	21.8	7.5	171	198
	6	258	32.8	3.31	72.5	32.2	12.8	295	125
	12	350	75.2	9.52	320	57.8	27.2	915	165

Fig. 2. Formation in relation to age of chlorophyll, plastoquinone and ubiquinone by autotrophically grown cells of *E. gracilis* strain Z. ○, Chlorophyll; △, plastoquinone; □, ubiquinone; ●, dry wt. of cells.

plastoquinone concentrations increased with age until active growth ceased (Table 5 and Fig. 2). This increase in quinone concentration with age has been noted for higher plants, e.g. oak leaves (Hindberg & Dam, 1965). The mean value for 13 analyses carried out on plastoquinone concentration between the sixth and tenth days, 1916 $\mu\text{g./g. dry wt.}$ (range 1348–2480 $\mu\text{g./g. dry wt.}$), was markedly higher than the value (1.11 mg./g. dry wt.) recorded by Fuller *et al.* (1961).

Ubiquinone, in all cases, showed a slight increase in concentration throughout the growth period, but varied little in concentration between the media used.

One noteworthy observation arising out of the study was the concentration of the $\Delta^{5,7}$ -sterol ester with respect to plastoquinone. In young autotrophic cultures the plastoquinone-containing fraction had a u.v. spectrum almost identical with that of authentic plastoquinone (Fig. 3), but in old cultures (8–9 days) the spectrum resembled the one shown in Fig. 1, suggesting that old cultures are accumulating

the $\Delta^{5,7}$ -sterol ester. In heterotrophic cultures plastoquinone was always accompanied by the $\Delta^{5,7}$ -sterol ester irrespective of the age of the culture.

Identification of the quinones occurring in E. gracilis. During the above studies it had become apparent that *E. gracilis* strain Z, in addition to containing plastoquinone, ubiquinone and possibly phylloquinone, also contained a further quinone that was eluted with the unesterified $\Delta^{5,7}$ -sterol (20% E/P) and that, from its difference spectrum, appeared to be α -tocopherolquinone. To characterize this and other quinones more fully, and also to check for the possible occurrence of plastoquinone B, plastoquinone C and plastoquinone D, a large culture of the organism was examined. At the same time etiolated cultures were examined for the presence of these quinones.

(a) Autotrophic cultures. A 71. volume of autotrophically grown *E. gracilis* strain Z was harvested after 7 days' growth; at this stage the $\Delta^{5,7}$ -sterol (free and esterified) does not interfere seriously with

Table 6. *Initial chromatography of lipid extract from E. gracilis strain Z*

A sample (26 g. wet wt., 5.3 g. dry wt.) of cells gave 995 mg. of lipid with a chlorophyll content of 229 mg. The extract was chromatographed on a column of 80 g. of acid-washed Brockmann grade III alumina (Woelm). Experimental conditions are given in the text.

Fraction no.	Eluent	Vol. (ml.)	Wt. (mg.)	Appearance of fraction and comments
1	0% E/P	800	9.0	Orange oil; fraction contains β -carotene (8.37 mg.) and phyloquinone, which at this stage is spectroscopically undetectable
2	1% E/P	800	26.3	Yellow oil; plastoquinone (8.38 mg.), $\Delta^{5,7}$ -sterol ester and carotenes present
3	3% E/P	800	6.8	Lemon-yellow oil; tocopherol (3.15 mg. estimated as α -tocopherol) present
4	5% E/P	800	9.1	Yellow oil; containing a trace of tocopherol (0.21 mg. as α -tocopherol) and ubiquinone (0.909 mg.)
5	8% E/P	800	4.2	Yellow oil: no selective absorption
6	12% E/P	800	12.0	Orange oil: no selective absorption
7	20% E/P	800	11.3	Brown and white solid; spectrum suggests presence of α -tocopherolquinone (2.94 mg.) and a $\Delta^{5,7}$ -sterol

Recovery: 78.7 (79%)

Fig. 3. Ultraviolet spectra of compounds purified by thin-layer chromatography. —, Plastoquinone (in ethanol); — $\cdot$ —, after treatment with NaBH_4 ; ----, phylloquinone (in cyclohexane); — $\cdot$ —, α -tocopherol (in cyclohexane).

the isolation of the quinones, and the total lipid was extracted by normal procedures. Table 6 summarizes details of the initial chromatography. Fraction 1 was rechromatographed on Brockmann grade II alumina and separated into two components: β -carotene (1% E/P) and a compound (2% E/P) (108 $\mu\text{g.}$) with a u.v. spectrum (Fig. 3) identical with that of phylloquinone (λ_{max} 243, 248, 260.5 and 270 $\text{m}\mu$ in cyclohexane). It behaved as phyloquinone on all reversed-phase paper- and thin-layer-chromatographic systems examined.

Fraction 2 contained plastoquinone, sterol esters and traces of carotenoids. The plastoquinone

(6 mg.) was purified as described above (Fig. 3) and recrystallized twice from 1 ml. of hot ethanol at room temperature overnight; it had m.p. 47° (uncorr.) and $E_{1\text{cm}}^{1\%}$ 240 at 254 $\text{m}\mu$; authentic plastoquinone-9 had m.p. 47° and $E_{1\text{cm}}^{1\%}$ 253 at 254 $\text{m}\mu$. The i.r. spectra (potassium bromide disk) of both specimens were identical.

Fraction 3 appeared to contain α -tocopherol (3.15 mg. by the modified Emmerie-Engel estimation). Thin-layer chromatography on Kieselgel G with benzene-chloroform (1:1, v/v) as developer yielded an Emmerie-Engel-staining spot with absorption bands at 292 and 297 $\text{m}\mu$ in cyclohexane (Fig. 3) identical with those of authentic α -tocopherol (Pennock *et al.* 1962). On thin-layer-chromatographic systems that will separate α -, β -, γ - and δ -tocopherol, only one spot, corresponding to α -tocopherol was observed.

Fraction 4 contained both α -tocopherol (210 $\mu\text{g.}$ by Emmerie-Engel estimation) and ubiquinone (909 $\mu\text{g.}$ estimated as ubiquinone-9). They were separated by thin-layer chromatography on Kieselgel G with benzene as solvent. Again only α -tocopherol was detected. The recovered ubiquinone (for u.v. spectrum see Fig. 4) was run on reversed-phase systems known to separate the ubiquinone isoprenologues; it ran with authentic ubiquinone-9. Further, the perhydro derivative, prepared by micro-dehydrogenation with Adams catalyst, co-chromatographed in these systems with authentic perhydroubiquinone-9. No evidence was obtained for the occurrence of ubiquinone-8 reported by Fuller *et al.* (1961) in *E. gracilis* strain Z.

Fractions 5 and 6 did not contain quinonoid material and were not further investigated.

Fraction 7 from its u.v. spectrum appeared to contain α -tocopherolquinone (estimated as 2.9 mg. by the sodium borohydride reduction method) and a

$\Delta^5,7$ -sterol. Purification by thin-layer chromatography on Kieselgel G with ethyl acetate-benzene (3:22, v/v) as developing solvent revealed two spots that stained with leucomethylene blue, with R_f values 0.42 and 0.72 respectively. The first had a u.v. spectrum (Fig. 4) identical with that of authentic α -tocopherolquinone prepared by gold chloride oxidation of α -tocopherol (Dilley & Crane, 1963), and behaved in the same way as α -tocopherolquinone on all chromatographic systems examined. Control experiments showed that it was not an

artifact. The second spot (constituting about 5% of the reducible components in the original fraction) was probably plastoquinone C or, less likely, plastoquinone D (Threlfall *et al.* 1965). It was not examined further. Plastoquinone B and plastoquinone D, which can be obtained from green leaves by the same procedures (Griffiths, 1965), were never detected.

A similar examination of other strains of *E. gracilis* yielded identical results, but quantitative differences (Table 7) were observed. This is particularly marked with plastoquinone, α -tocopherol and α -tocopherolquinone. Values for chlorophyll and β -carotene are also included in Table 7 for comparative purposes.

Threlfall *et al.* (1965) on the basis of limited distribution studies have proposed three types of quinone pattern in the chloroplast. The first and simplest, to which *Euglena* belongs, is characterized by the occurrence of phyloquinone, plastoquinone, α -tocopherolquinone (and α -tocopherol). The other two consist of these quinones plus either plastoquinone C and plastoquinone D, or plastoquinone B, plastoquinone C and plastoquinone D.

Powls & Hemming (1966*a,b*) reported that *E. gracilis* var. *bacillaris* contains the aminoquinone rhodoquinone. This quinone possesses a nonaprenyl side chain and is apparently a metabolite of ubiquinone-9. Our investigations failed to show the presence of this compound. The probable explanation for our failure to observe this quinone, which on preliminary chromatography on alumina would be eluted by 12% E/P (Whistance, Threlfall & Goodwin, 1966), is due to the fact that, on treatment of the appropriate fraction with sodium borohydride, our standard method at this time for the detection

Fig. 4. Ultraviolet spectra of compounds purified by thin-layer chromatography. —, Ubiquinone (in ethanol); — — —, after treatment with NaBH_4 ; — — —, α -tocopherolquinone (in ethanol); — — —, after treatment with NaBH_4 .

Table 7. Concentrations of terpenoid quinones, α -tocopherol, chlorophyll and β -carotene in various strains of *E. gracilis*

Cells from 7-day-old autotrophically grown cultures were examined by the procedures outlined in the text. Each analysis was carried out on the cells obtained from 5l. of culture medium for strain Z and 1l. of culture medium for var. *bacillaris* and strain 1224/5g. N.D., Not determined.

Strain	Concn./g. dry wt.								
	Dry wt. (mg./ 100ml.)	Lipid (mg./g. dry wt.)	Chloro- phyll (mg.)	β - Carotene ($\mu\text{g.}$)	Phyllo- quinone ($\mu\text{g.}$)	Plasto- quinone ($\mu\text{g.}$)	α -Toco- pherol ($\mu\text{g.}$)	α -Toco- pherol- quinone ($\mu\text{g.}$)	Ubi- quinone-9 ($\mu\text{g.}$)
Strain Z	106	187	43.2	1580	20	1580	634	555	171
	104	190	40.9	2400	Present	1890	623	308	182
	120	220	44.2	N.D.	Present	2480	846	401	224
Var. <i>bacillaris</i>	98	200	40.7	840	Present	691	435	440	260
	86	196	28.4	760	Present	662	320	260	199
Strain 1224/5g	62	182	39.8	1670	Present	1344	340	79*	182
	50	176	42.4	2000	Present	1520	410	212	196

* Unexpectedly low.

Table 8. *Terpenoid quinone concentration relative to chlorophyll in Euglena and other photosynthetic tissues*

Values for the *E. gracilis* strains are calculated from the mean of the values given in Table 7. Values for spinach chloroplasts, broad-bean chloroplasts and tobacco leaves are taken from Threlfall & Griffiths (1966). Values for maize shoots and barley shoots are taken from Griffiths *et al.* (1967).

Component	Concn. (μ moles/100 μ moles of chlorophyll)						Maize shoots (7 days old)	Barley shoots (5 days old)
	<i>E. gracilis</i> strain Z	<i>E. gracilis</i> var. <i>bacillaris</i>	<i>E. gracilis</i> strain 1224/5g	Spinach chloroplasts	Broad- bean chloroplasts	Tobacco leaves		
Chlorophyll	100	100	100	100	100	100	100	100
β -Carotene	7.8	3.9	7.5	8.3	4.8	8.9	1.2†	3.0
Phylloquinone	0.1	Present	Present	1.2	0.8	1.4	0.24	Present
Plastoquinone	5.5	2.4	4.2	10.0	4.8	12.2	2.7	4.5
α -Tocopherolquinone	2.0	2.0	1.0*	0.7	1.6	1.5	1.7	1.2

* Highest value in Table 7 used in calculating this value.

† Lower than expected.

of quinones, rhodoquinone is rapidly reoxidized after reduction (Threlfall, 1962) and we would fail to detect any spectroscopic changes indicative of the presence of a quinonoid compound.

The concentrations of the chloroplastidic quinones (phylloquinone, plastoquinone and α -tocopherolquinone) relative to each other are similar to those found in other plant tissues (Table 8).

(b) Etiolated cultures. Fuller *et al.* (1961) were unable to detect any plastoquinone in dark-grown *Euglena* cells; ubiquinone, on the other hand, was present in significantly higher amounts than in the corresponding autotrophic cultures. Since the former observation obviously had a bearing on chloroplast development and formation, cultures of etiolated *E. gracilis* strain Z were grown on the heterotrophic medium (Wolken, 1961) in the dark and transferred three times before analysis. The only compounds detected were ubiquinone-9 and α -tocopherol; ubiquinone was present at concentrations similar to those in light-grown cultures (see above); however, α -tocopherol was present at only one-sixth the concentration found in green cultures. Table 9 records the results of a series of typical experiments. Analysis of the cultures was difficult, owing to the presence of large amounts of a wax that ran in the early fractions eluted off the column. On the basis of i.r. studies and chromatographic behaviour this material corresponds to the myristyl myristate-containing fraction described by Rosenberg (1963), which can contribute up to 50% of the weight of lipid in etiolated cultures.

Etiolated tissues of normally photosynthetic regions of plants usually contain recognizable structures (proplastids) that on illumination will give rise to chloroplasts; in addition, it is possible to detect some of the terpenoid quinones associated

Table 9. *Components of etiolated E. gracilis strain Z*

The organism was grown on the medium of Wolken (1961), which contains glucose (1%) and organic acids. The culture was taken through four transfers before the analysis was carried out. Eight-day-old cells from 400 ml. of medium containing about 200 mg. dry wt. of cells/100 ml. were examined in each case.

Component	Concn. (μ g./g. dry wt.)		
	Expt. I	Expt. II	Expt. III
Phylloquinone, plastoquinone and α -tocopherolquinone	0	0	0
α -Tocopherol	98	140	111
Ubiquinone-9	220	200	250

with the mature chloroplast. Griffiths *et al.* (1967) have shown that etiolated maize shoots (which have well-developed proplastids) contain lower amounts of phylloquinone and plastoquinone and that α -tocopherolquinone is absent. In etiolated *Euglena* cells, where it is not possible to identify any structures that on illumination will give rise to chloroplasts (Wolken & Palade, 1953), no evidence could be found for the occurrence of phylloquinone, plastoquinone or α -tocopherolquinone. Even so, α -tocopherol, which from sedimentation-distribution studies on green cells appears to be mainly localized in the chloroplast (Table 10 and Fig. 5), was detected in etiolated cultures (Table 9). This suggests that in etiolated and autotrophic cells some α -tocopherol can be associated with structures other than the chloroplast.

Intracellular distribution of plastoquinone, ubiquinone, α -tocopherolquinone and α -tocopherol.

Studies on leaf tissues have shown that phyloquinone, plastoquinone, α -tocopherolquinone and α -tocopherol are localized wholly within the chloroplast, whereas ubiquinone is found elsewhere in the plant cell, i.e. the mitochondria. It was obviously desirable to test whether a similar situation held true for *E. gracilis*. A number of fractionations were carried out, of which we report two here, with 5½- and 8-day-old autotrophically grown cultures. Since the main intention of the experiments was to show the intra- or extra-chloroplastidic localization of the various compounds under investigation a rigorous purification procedure was not considered necessary. It appeared that the best approach was to carry out a sedimentation-distribution study and then to extrapolate the components under investigation with compounds (e.g. chlorophyll) known to occur only in the chloroplast. The results obtained are given in Table 10 and Fig. 5.

The values show that the preparations obtained by ultrasonic treatment of the *Euglena* cells reflect the situation in whole cells. Further, the total

recoveries of all components from the various fractions are in good experimental agreement with the amounts present in the original preparations.

By taking chlorophyll as a measure of the chloroplasts or chloroplast fragments occurring in any one fraction it is clear (Table 10 and Fig. 5) that for the particulate fractions plastoquinone, α -tocopherolquinone and α -tocopherol concentrations parallel chlorophyll distribution. Ubiquinone, as expected, showed a distribution pattern consistent with its localization in the mitochondria. Two points that require further comment are, first, the presence in the supernatant of plastoquinone and α -tocopherol in the 5½-day-old cells, and of plastoquinone and α -tocopherolquinone in the 8-day-old cells, without the concomitant occurrence of chlorophyll. Two explanations have been offered for this phenomenon: Threlfall & Goodwin (1964) suggested that the presence of these compounds in the supernatant may be due to the liberation of osmiophilic globules (Bailey & Whyborn, 1963) containing plastoquinone, α -tocopherolquinone and α -tocopherol from the chloroplast during the ultrasonic treat-

Table 10. *Intracellular distribution of plastoquinone, ubiquinone, α -tocopherolquinone and α -tocopherol in autotrophic E. gracilis strain Z*

Autotrophically grown cells were suspended in sucrose solution (10%, w/v) and treated ultrasonically. The resulting preparation after removal of (a) intact cells by centrifugation and (b) a sample for analysis was subjected to a scheme of differential centrifugation. The degree of cell rupture, as measured by chlorophyll content, was 28% for the 5½-day-old cells and 21% for the 8-day-old cells. Fig. 5 gives the percentage distribution between fractions of the components examined in the 5½-day-old cells. The distribution pattern for the 8-day-old cells was essentially similar.

Cell fraction	5½-day-old cells					8-day-old cells				
	Chlorophyll (mg.)	Plastoquinone (µg.)	α -Tocopherolquinone (µg.)	α -Tocopherol (µg.)	Ubiquinone-9 (µg.)	Chlorophyll (mg.)	Plastoquinone (µg.)	α -Tocopherolquinone (µg.)	α -Tocopherol (µg.)	Ubiquinone-9 (µg.)
Whole cell (corrected)*†	10	625	111	219	61	10	416	84	194	37
Preparation (corrected)†	10	599	113	203	55	10	398	84	177	30
Preparation (actual)	17.7	1060	200	359	97	22.7	804	190	402	68
Fractionation:										
3000g ('chloroplasts')	12.1	617	124	180	16.2	14	499	132	338	18
20000g ('mitochondria')	3.1	115	41	48	51.9	5.9	121	35	82	34
105000g ('microsomes')	1.5	78	17	24	19.4	1.1	26	8	12	8
Supernatant	0.03	186	13	94	3.2	0.0	104	11	10	0
Recovery:	16.73 (95%)	996 (94%)	195 (98%)	346 (97%)	90.7 (93%)	21.0 (93%)	750 (93%)	186 (98%)	442 (110%)	60 (88%)

* Actual amounts of chlorophyll in the two samples were 62.7 mg. for the 5½-day-old cells and 108 mg. for the 8-day-old cells.

† Both sets of values are expressed on the basis of 10 mg. of chlorophyll for the sake of comparison.

ment; Stevenson, Hemming & Morton (1963), working with tobacco leaves, proposed that the occurrence of plastoquinone in the supernatant fraction was indicative of the presence of small underdeveloped plastids. The second point requiring comment is that, although the distribution studies are consistent with the occurrence of the greater part of the total α -tocopherol in the chloroplast, evidence has been obtained on dark-grown cultures that some α -tocopherol may be associated

with structures other than the chloroplast (see above).

Effect of white light on the synthesis of plastoquinone, α -tocopherolquinone and α -tocopherol by etiolated non-dividing cultures of E. gracilis. The finding that plastoquinone, α -tocopherolquinone and α -tocopherol are associated with the chloroplast prompted an investigation into the formation of these compounds by etiolated *Euglena* cells exposed to the light. Such cells, which contain no chloroplasts, will on illumination form functional chloroplasts (Brawerman & Chargaff, 1959).

Heterotrophic cultures [medium of Pringsheim & Pringsheim (1952) + 0.3% sodium acetate] (4×750 ml.) were inoculated from an 8-day-old etiolated culture that had been obtained by two transfers of autotrophically grown cells through a rich heterotrophic medium (Wolken, 1961) in the dark; the flasks, wrapped in tinfoil, were kept in the dark for $5\frac{1}{2}$ days. A control autotrophic culture was inoculated at the same time.

At the end of this period the cultures of etiolated cells were collected by low-speed centrifugation, washed several times with sterile distilled water and suspended in a 'resting medium' (one-fifth of the original culture volume), which was that of Brawerman & Chargaff (1959) with the omission of glucose, since this compound suppresses the rate of greening (Smillie, 1963). The cells were then distributed among eight 250ml. conical flasks. All manipulations were carried out under aseptic conditions and in subdued light. The flasks were then placed 1 ft. from a fluorescent strip-light and gently agitated. Flasks were taken at 0, 3, 6, 12, 24, 48, 72 and 96 hr. for examination. The control green culture was analysed at the same time as the zero-time culture.

Fig. 5. Percentage distribution of chlorophyll, plastoquinone, α -tocopherolquinone, α -tocopherol and ubiquinone among the fractions obtained from differential centrifugation of an extract prepared by ultrasonic treatment of autotrophically grown cells of *E. gracilis* strain Z. O, Chlorophyll; Δ , plastoquinone; $\square$, ubiquinone; $\bullet$, α -tocopherol; $\blacktriangle$, α -tocopherolquinone.

Table 11. *Effect of white light on the synthesis of plastoquinone, α -tocopherolquinone, α -tocopherol and ubiquinone by etiolated cultures of E. gracilis strain Z*

Dark-grown cells from 4l. of growth medium [that of Pringsheim & Pringsheim (1952) + 0.3% sodium acetate] were resuspended in the medium (8×100 ml.) of Brawerman & Chargaff (1959) (glucose was omitted), which does not support cell division, and exposed to white light. Samples (100 ml.) were then taken for chlorophyll, plastoquinone, ubiquinone, α -tocopherolquinone and α -tocopherol determinations at appropriate time-intervals. Fig. 6 records the result for the whole experiment.

Time in light (hr.)	Concn./g. dry wt.					
	Chlorophyll (mg.)	Plastoquinone (μ g.)	α -Tocopherolquinone (μ g.)	α -Tocopherol (μ g.)	α -Tocopherol + α -tocopherolquinone* (μ g.)	Ubiquinone-9 (μ g.)
Control†	40.9	1890	308	623	919	182
0‡	0	19	0	156	156	234
72‡	15.2	707	154	375	523	169
96	14.6	694	120	326	442	181

* Expressed as the chromanol.
† $5\frac{1}{2}$ -day-old autotrophically grown culture.
‡ Intermediate values given in Fig. 6.

Fig. 6. Effect of white light on the formation of chlorophyll, plastoquinone, α -tocopherolquinone, α -tocopherol and ubiquinone by etiolated (dark-grown) cells of *Euglena*. $\circ$, Chlorophyll; Δ , plastoquinone; $\square$, ubiquinone; $\bullet$, α -tocopherol; $\blacktriangle$, α -tocopherolquinone.

Table 11 gives the control, initial and final (0, 72 and 96 hr.) values for the experiment, and Fig. 6 gives a graphical presentation of the complete experiment.

In the interpretation of the results obtained from this experiment the concentration of chlorophyll was used as a measure of chloroplast development. The results show that the etiolated cultures did contain traces of some quinones and the chromanol normally associated with chloroplasts. Since in previous analyses of etiolated cultures we failed to find plastoquinone and α -tocopherolquinone, although α -tocopherol was detected, these results suggest that insufficient dark transfers were carried out to deplete the cells of all the chloroplast components from the original autotrophic inoculation.

Fig. 6 shows that during the first 6 hr. of illumination all the components increased in terms of

absolute concentration (per g. dry wt.), although this may be only an apparent increase owing to variation in the non-lipid component of the cells, such as carbohydrate. This would seem to be supported by the observations of App & Jagendorf (1963), who found that etiolated cells placed in the light without a carbon source, or with one they cannot utilize efficiently, are forced to use storage material for endogenous metabolism and chloroplast synthesis. As photosynthesis begins, storage material may begin to accumulate.

At 24 hr. plastoquinone, α -tocopherolquinone and α -tocopherol were being actively synthesized in step with chlorophyll and hence chloroplast development; this trend continued up to 72 hr., when synthesis began to fall away. The ubiquinone concentration, after rising initially, slowly declined to the normal. The concentrations of the com-

ponents measured in the greened etiolated cells failed to reach the values obtained for the control cultures (5½ days old).

The formation of the chloroplastidic components in these experiments followed closely the morphological changes observed by Wolken & Palade (1953) for this system. These workers found no evidence for chloroplast structures in dark-grown *Euglena*; however, after 4 hr. of illumination elongated bodies with the characteristic lamination of chloroplasts were just discernible, and after 72 hr. of illumination structures showing the form and organization found in green cells were present.

Incorporation of [2-¹⁴C]mevalonic acid into E. gracilis. It had been intended to use *Euglena* to study the biosynthesis of the prenyl portions of terpenoid quinones by this organism. Steele & Gurin (1960) had found that [2-¹⁴C]mevalonic acid was incorporated into β -carotene by heterotrophic cultures of *E. gracilis* var. *bacillaris*, although the incorporation was poor, namely, 0.022–0.060%. In numerous experiments with three strains of *E. gracilis* (Z, var. *bacillaris* and 1224/5g) and under a variety of experimental conditions we have failed to observe any significant incorporation of [2-¹⁴C]mevalonic acid into the terpenoids by this organism. We attribute this failure to the impermeability of the organism's cell wall to [2-¹⁴C]mevalonic acid rather than to a novel pathway of terpenoid biosynthesis.

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