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THE PROPERTIES AND SIGNIFICANCE OF RHODOQUINONE-9 IN AUTOTROPHIC AND ETIOLATED CULTURES OF *EUGLENA GRACILIS* VAR. *BACILLARIS**

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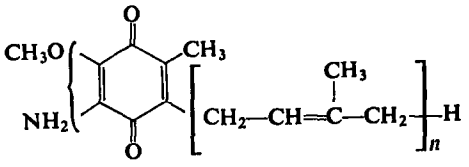
Abstract—A new quinone isolated from *Euglena gracilis* was characterized, using principally spectrometric techniques, as rhodoquinone-9. The level of this compound was similar in both autotrophic and etiolated cultures suggesting that it did not function in photosynthesis. The evidence suggests that rhodoquinone is probably of mitochondrial origin. Also the absence of rhodoquinone and ubiquinone and the presence of "plastidic quinones" in *Anabaena variabilis* is contrasted with the reverse situation in another procaryotic, photosynthetic micro-organism *Rhodospirillum rubrum*.

INTRODUCTION

THE isolation and partial characterization of rhodoquinone from *Rhodospirillum rubrum* was first described by Glover and Threlfall.[1] Further work by Moore and Folkers[2] has shown the compound to be the derivative of ubiquinone-10 in which one of the methoxyl groups is replaced by an amino group (I, $n=10$).



(I)

The elegant work of Parson and Rudney[3] has made it quite clear that in *R. rubrum*, rhodoquinone is formed from ubiquinone. Since *R. rubrum* is a photosynthetic micro-organism, Moore and Folkers[2] have suggested a possible role for rhodoquinone in photosynthesis.

The present paper reports the isolation, properties and characterization of a shorter isoprenologue of rhodoquinone, rhodoquinone-9, from dark-grown, heterotrophic, etiolated cultures as well as from light-grown, autotrophic cultures of *Euglena gracilis*. The relevance of this finding to a possible role of the quinone in photosynthetic electron transport is discussed. The biosynthetic relationship between rhodoquinone-9 and ubiquinone-9 in this organism is considered elsewhere.[4]

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[1] J. GLOVER and D. R. THRELFALL, *Biochem. J.* **85**, 14p (1962).
[2] H. W. MOORE and K. FOLKERS, *J. Am. Chem. Soc.* **87**, 1409 (1965).
[3] W. W. PARSON and H. RUDNEY, *J. Biol. Chem.* **240**, 1853 (1965).
[4] R. POWLS and F. W. HEMMING, *Phytochem.* **5**, 1249 (1966).

RESULTS AND DISCUSSION

The level of the new quinone is about the same (250–300 μg/g dry wt.) in both autotrophic and heterotrophic cultures of *E. gracilis* when these are harvested in the late log phase. However, it is more convenient to grow the organism in bulk under heterotrophic conditions and in fact the yield of organism from each litre of medium is considerably higher than when grown under autotrophic conditions. For these reasons *E. gracilis* was grown in the dark in forty (separate) litres of heterotrophic medium over 5 days. The harvested organism was lyophilized and the extracted lipid was chromatographed on columns of silicic acid–celite (2:1). Further chromatographic purification on thin layers of silica gel G, followed by crystallization from ethanol yielded a pure specimen of the quinone. The compound crystallized in spherical clusters of tightly packed needles, magenta in colour and which melted at 66·5–67 .

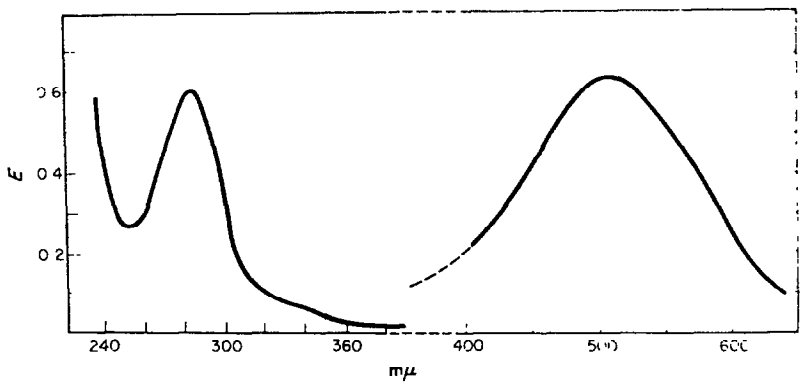


FIG. 1. THE ABSORPTION SPECTRUM OF THE *Euglena* QUINONE IN ETHANOL.
240–400 nm, 4·285 mg %, 400–630 nm, 36·6 mg %.

Ultra-violet Absorption

The u.v. absorption spectrum of an ethanolic solution of the quinone is shown in Fig. 1. The $E^{1\%}_{1cm}$ values at $\lambda\lambda_{max}$ 283 and 506 nm are respectively 140 and 17·8 with that at λ_{min} 253 nm being 61·1. The positions of these maxima and minimum agree well with those of rhodoquinone-10 ($\lambda\lambda_{max}$ 283, 500 nm, λ_{min} 253 nm[1]) but the $E^{1\%}_{1cm}$ values are a little higher than those of rhodoquinone-10 ($E^{1\%}_{1cm}$ 126·2 at 283 nm[3]). Both the *Euglena* quinone and rhodoquinone-10 gave $\lambda\lambda_{max}$ at 279 nm and 506 nm with λ_{min} at 252 nm. when dissolved in cyclohexane.

Treatment of an ethanolic solution of the compound with sodium borohydride followed by saturation of the solution with nitrogen resulted in the peak at 283 nm being replaced by a new peak at 290 nm. The intensity of this new peak was about 50 per cent of the original peak at 283 nm. The reduced material was colourless, the peak at 506 nm having disappeared. This change in u.v. absorption is characteristic of the reduction of rhodoquinone-10.[3] When the solution was not saturated with nitrogen immediately after borohydride treatment, the rapid appearance of a peak at 290 nm (after 30 sec) was followed by a gradual rise in intensity and shift of the peak. After 4 min λ_{max} was at 284 nm ($E^{1\%}_{1cm}$ 118) and the purple colour had reappeared. The change continued, to give λ_{max} at 289 nm ($E^{1\%}_{1cm}$ 145) after 22 min and λ_{max} at 295 nm ($E^{1\%}_{1cm}$ 211) after 50 min. A series of spectral changes of this

sort for rhodoquinone was first described by Threlfall.[5] While the initial effect of borohydride is undoubtedly due to reduction of quinone to quinol, it seems clear that the further spectral changes are due to phenomena more complex than, or in addition to, the simple reoxidation of quinol to quinone suggested by Parson and Rudney.[3]

The u.v. absorption spectrum obviously indicates the presence of the same chromophoric group in the *Euglena* quinone as is present in rhodoquinone-10. The intensity of the spectral maxima suggests that the molecular weight of the quinone is a little smaller than that of rhodoquinone-10.

Infra-red Absorption

The i.r. absorption spectrum of the *Euglena* quinone was determined as a potassium bromide disc and compared with that of ubiquinone-10, also studied as a potassium bromide disc. The spectrum of the *Euglena* quinone is recorded in Fig. 2. The probable assignments of the main areas of absorption are listed in Table 1. The presence of an NH_2 group is confirmed by the presence of peaks at 3478 and 3362 cm^{-1}. Infrared absorption due to N—H deformation probably explains why absorption in the 1610–1615 cm^{-1} region was stronger in the spectrum of the *Euglena* quinone than in the spectrum of ubiquinone. Pennock[6] has summarized the arguments for and against the absorption band at 1266 cm^{-1}

TABLE 1. INFRARED ABSORPTION OF THE *Euglena* QUINONE AND OF UBIQUINONE-10

Positions of absorption bands (cm^{-1})		
Euglena quinone	Ubiquinone-10	Probable assignments
3478		N—H stretching[7]
3362		
2850–3050	2850–3050	C—H stretching and deformation[7]
1650	1653	Quinone C=O stretching[6]
1610	1615	Quinone C=C stretching[6]
1610		N—H deformation[7]
1580~		
1445	1445	Methyl C—H asymmetrical deformation[7] and methylene C—H deformation[7]
1380	1380	Methyl C—H symmetrical deformation[7]
1350		?
1309		C—N stretching[7]
1285	1290	Associated with quinone group?[6]
	1266	?
1209	1205	O—CH_3 vibration[6]
1149	1152	O—CH_3 vibration[6]
1103	1100	O—CH_3 vibration?[6]
	1015	?
995		?
940		?
872	872	Crystallization bands of tri-substituted ethylenes[6]
785	785	
740	740	

[5] D. R. THRELFALL, Ph.D. Thesis, University of Liverpool (1962).
[6] J. F. PENNOCK, In *Biochemistry of Quinones* (Edited by R. A. MORTON), p. 67. Academic Press, New York (1965).
[7] L. J. BELLAMY, *The Infra-red Spectra of Complex Molecules*. Methuen, London (1958).

in the spectrum of ubiquinone being due to the presence of methoxyl groups. The fact that this band is absent from the spectrum of rhodoquinone-10[5] and from the *Euglena* quinone is evidence against it being due to CH_3—O vibrations. The similar patterns of absorption of the quinone and of ubiquinone in the regions 2830–3050, 1380–1445 and 740–872 cm^{-1} is consistent with the presence of a long polyisoprenoid side-chain in the *Euglena* quinone.

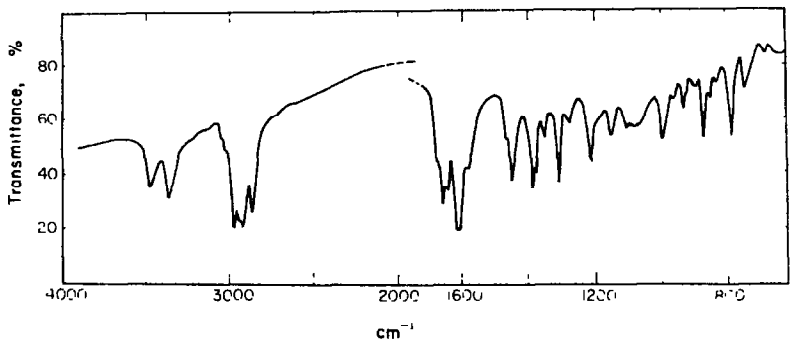


FIG. 2. THE I.R. ABSORPTION SPECTRUM OF THE *Euglena* QUINONE (KBr DISC).

Nuclear Magnetic Resonance

The NMR spectrum of 20 mg of the quinone in carbon tetrachloride was determined at 60 Mc/s; Table 2 records the relevant data. It can be seen that the spectrum is reasonably

TABLE 2. NUCLEAR MAGNETIC RESONANCE DATA FOR THE *Euglena* QUINONE

Position of peak (τ)	Relative area of peak	Assignment* of peak	Expected relative areas for: RQ-9†	RQ-10†
4·90	8·7	$-\underline{CH}=\overset{\displaystyle|}{C}-$	9	10
5·52	1·5	ring $-N\underline{H}_2$	2	2
6·12	3·0	ring $-O\underline{CH}_3$	3	3
6·80	1·5	ring $-C\underline{H}_2-$	2	2
7·93	33·9	ring $-C\underline{H}_3$ + side chain $-C\underline{H}_2-$'s	35	39
8·17	32·4	*trans* $-CH=\overset{\displaystyle\underline{CH}_3}{\overset{\displaystyle|}{C}}-$ next to ring[6]	3	3
8·28 (shoulder)		*cis*, terminal $-CH=\overset{\displaystyle\underline{CH}_3}{\overset{\displaystyle|}{C}}-$ [6]	3	3
8·40		*trans*, $CH=\overset{\displaystyle\underline{CH}_3}{\overset{\displaystyle|}{C}}$ [6]	24	27
Total areas	81·0		81	89

* The resonating proton is underlined.
† RQ— rhodoquinone abbreviated.

consistent with the compound being rhodoquinone-9. The spectrum shows that the polyisoprenoid side-chain is all *trans*. The shoulder at 8·28 τ was of the same intensity as the 8·17 τ peak, the area of which was close to 10 per cent of that of the combined areas of the peak at 8·40 τ and the shoulder at 8·28 τ. This agrees with the presence of only one methyl *cis* to an olefinic proton, namely one of the two methyl groups in the terminal isoprene unit. The positions of the peaks reported in Table 2 are close to those previously reported for rhodoquinone-10.[2]

Reversed-phase Partition Chromatography

Small quantities of rhodoquinone-9 and rhodoquinone-10 were prepared by ammonolysis[2] of ubiquinone-9 and ubiquinone-10. Rhodoquinone-9 was also isolated from a culture of *R. rubrum*. The mobility of these three preparations when subjected to reversed-phase partition chromatography was compared with that of the *Euglena* rhodoquinone. The R_f of the *Euglena* rhodoquinone (0·64) was the same as that of synthetic rhodoquinone-9 but was different from that of synthetic rhodoquinone-10 (0·51). The *Rhodospirillum* rhodoquinone gave a major spot corresponding to rhodoquinone-10 and a small spot corresponding to rhodoquinone-9. It seems likely that of the rhodoquinone isolated from *R. rubrum*, approximately 5 per cent may be rhodoquine-9.

Mass Spectrometry

The final proof that the new *Euglena* quinone is in fact rhodoquinone-9 was provided by its mass spectrum. A histogram of the mass spectrum, in which the height of the peaks is represented as a percentage of the height of the base peak at $m/e=69$, is shown in Fig. 3. The intensities of the more prominent peaks and their most likely assignments are recorded in Table 3. A prominent molecular ion at $m/e=779$ occurred and a cracking pattern indicating consecutive loss of 69 (once) and then of 68 (seven times) was also quite clear. This is good evidence of the progressive loss of eight of the nine isoprene residues (including the terminal residue). Such a pattern is common to the mass spectra of ubiquinone, solanesol, castaprenols, dolichol (unpublished work) and of plastoquinones[8] and is that expected from rupture of the energetically relatively weak links between the adjacent methylene groups in the polyisoprenoid chains. The quinone ring influenced the rupture of the final isoprene residue at the bond β to the ring to leave one methylene group attached to the ring. The ion so produced appeared to be present as the quinol rather than as the quinone. Possibly the increased aromatization stabilized such an ion. It is relevant that ubiquinone-4 also gave a prominent peak ($m/e=197$) corresponding to the quinol carrying a methylene group instead of the isoprenoid side-chain (unpublished work). The mass spectra of plastoquinones also show quinol ions.[8] No prominent peaks resulting from the rupture of the rhodoquinone nucleus were observed. The more prominent of the low-mass peaks could be accounted for on the basis of their arising from the terminal unit of the side chain. The assignment of the peaks at $m/e=41$ and 81 requires migration of double bonds. The peaks at $m/e=41$, 55 69 and 81 are common to the mass spectra of ubiquinone, solanesol, castaprenols and dolichol (unpublished work).

Analysis

Euglena rhodoquinone analysed as C, 81·79; H, 10·81; N, 1·55%. The theoretical figures expected for rhodoquinone-9 are C, 81·53; H, 10·51; N, 1·76%.

[8] B. C. Das, M. Lounasmaa, C. Tendille and E. Lederer, *Biochem. Biophys. Res. Commun.* **21**, 318 (1965).

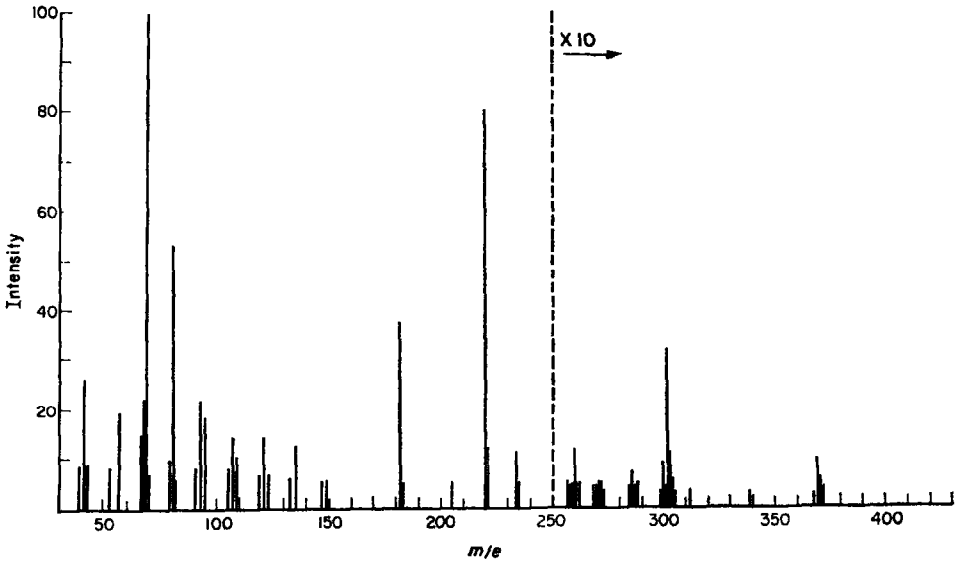


FIG. 3*a*.

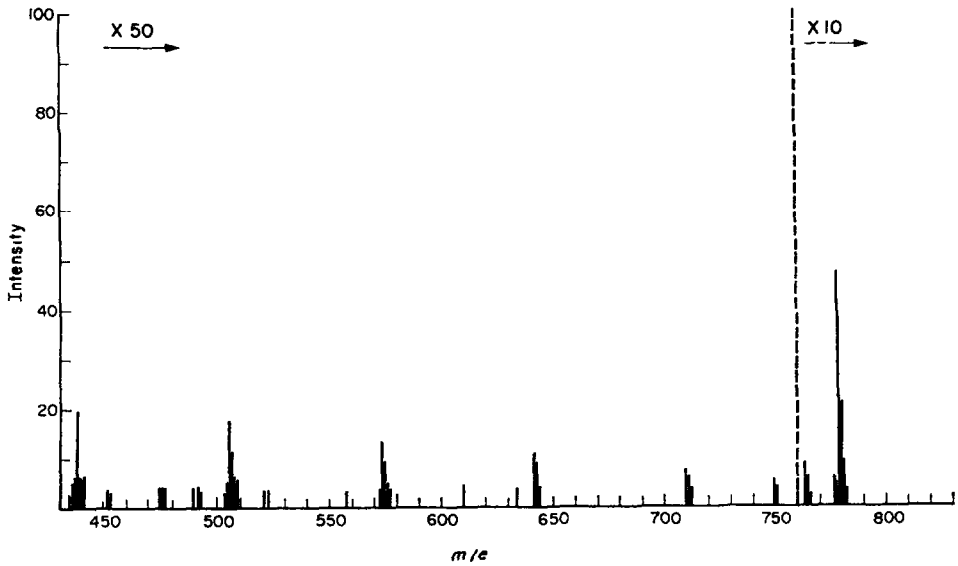
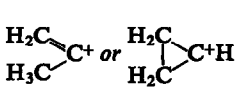
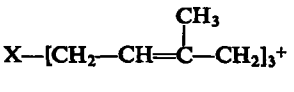
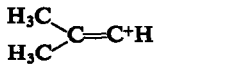
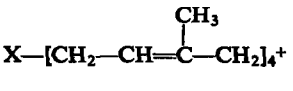
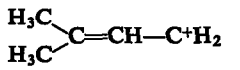
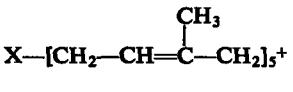
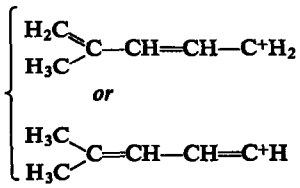
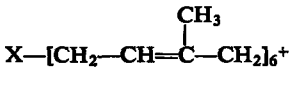
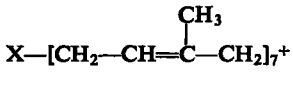
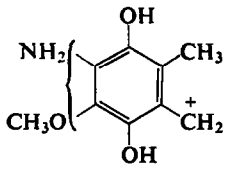
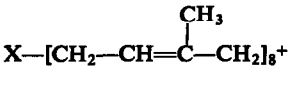
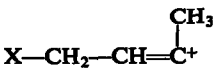
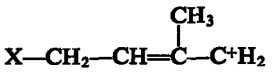
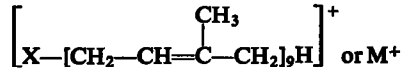
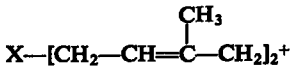


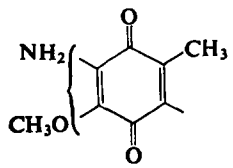
FIG. 3*b*.

FIG. 3. A HISTOGRAM OF THE MASS SPECTRUM OF THE *Euglena* QUINONE SHOWING THE INTENSITIES OF THE PEAKS RELATIVE TO THAT AT *m/e* 69 (ARBITRARY INTENSITY = 100).

Peaks of intensity less than 5% (*m/e* 30–250), 0·2% (*m/e* 250–430 and 760–830) and 0·04% (*m/e* 430–760) of that of the peak at *m/e* 69 have been omitted.

TABLE 3. ASSIGNMENTS AND RELATIVE INTENSITIES OF THE MORE PROMINENT PEAKS IN THE MASS SPECTRUM OF *Euglena* RHODOQUINONE

m/e	Intensity*	Assignment	*m/e*	Intensity*	Assignment
41	26		370	0·9	
55	19		438	0·4	
69	100		506	0·34	
81	53†		574	0·26	
			642	0·22	
182	37		710	0·14	
220	80		764	0·9	M^+ minus CH_3
234	11		779	4·7	 or M^+
302	3·2				

X = 

* Relative to *m/e* 69 = 100.
† Or a cyclic isomer thereof.

General Discussion of the Chemistry of Rhodoquinone-9

The evidence already discussed shows that the *Euglena* quinone is in fact rhodoqiunone-9 (I, $n=9$). The problem still remaining is the precise position of the amino- and methoxyl-groups.* Clearly there are two possible isomers. Moore and Folkers[2] have argued that since natural rhodoquinone-10 has a melting point of 69–70° and synthetic rhodoquinone-10 has a melting point of 39–45°, natural rhodoquinone-10 is probably just one of the isomers while the synthetic material is most likely a mixture of the two possible isomers. The melting point of 66·5–67° of *Euglena* rhodoquinone-9 supports the idea that this compound. also. is only one of the two possible isomers.

It seems that to determine which of the two isomers is present, one really needs to make the two possible positions for the amino- and methoxyl-groups markedly non-equivalent. The most obvious way of doing this would be to cause the compound to cyclize to the hypothetical compound rhodochromenol (Fig. 4). The product should then have some properties in common with either an *ortho*-amino phenol or a *meta*-amino phenol. These two compounds should be distinguishable on the basis of a number of well-known chemical tests and probably also by NMR.

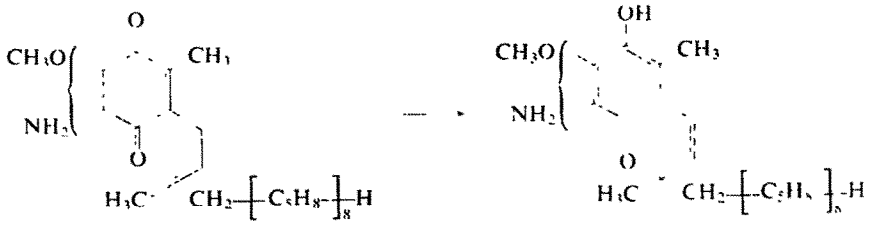


FIG. 4. THE HYPOTHETICAL CYCLIZATION OF RHODOQUINONE-9 TO RHODOCHROMENOL-8.

Probably the best method for converting ubiquinone to ubichromenol is that involving refluxing the quinone with pyridine.[9] This method was attempted with natural rhodoquinone-9 but unfortunately the yield was very poor and insufficient natural compound was available to produce the rhodochromenol required to carry out the appropriate chemical tests. Obviously, pin-pointing the precise position of the amino- and methoxyl-groups will be difficult.

TABLE 4. LEVELS OF SOME ISOPRENOID COMPOUNDS IN AUTOTROPHIC AND ETIOLATED CULTURES OF *E. gracilis* VAR. *bacillaris*

Compound	Levels (μg/g dry wt.)	
	Autotrophic cells	Etiolated cells
Plastoquinone-9	306	0
α-Tocopherolquinone	383	0
Ubiquinone-9	140	145
Phytoene	72	--
Phytofluene	7	—
α-Tocopherol	60	53

* In a very recent paper H. W. MOORE and K. FOLKERS (*J. Am. chem. Soc.* **88**, 567, 1966) have shown, by very elegant work involving NMR, that rhodoquinone-10 from *R. rubrum* has its amino group *para* and its methoxyl group *meta* to the isoprenoid side-chain.

[9] D. MCHALE and J. GREEN, *Chem. & Ind. (London)* 1867 (1962).

Levels of Rhodoquinone and Other Polyisoprenoid Compounds in Light- and Dark-grown Euglena

One of the most interesting aspects of the discovery of rhodoquinone-9 in *E. gracilis* is that the levels (250–300 μg/g dry wt.) of the compound are essentially the same in both light-grown, autotrophic cells and in dark-grown, heterotrophic etiolated cells. The latter type of cells do not contain chloroplasts. The levels of various other isoprenoid compounds in the two types of cells are reported in Table 4. It is not surprising to find that the plastidic quinones plastoquinone and α-tocopherol quinone could not be detected in etiolated cells whereas in light-grown cells both of these quinones were present at relatively high levels (see also Ref. 10). These plastidic quinones function in photosynthetic electron transport.[11] On the other hand the levels of ubiquinone-9 in the two types of cells were almost identical; a situation consistent with ubiquinone functioning in mitochondrial but not photosynthetic electron transport in *E. gracilis*. The similar levels of rhodoquinone in light- and dark-grown cultures of *Euglena* also point against a function for this quinone in photosynthetic electron transport in this organism. It is also relevant that rhodoquinone was not detected in the lipid extracted from chloroplasts, of *E. gracilis*, prepared by the non-aqueous method described by Smillie[12] whereas both plastoquinone-9 and α-tocopherol quinone were present.

It will be shown elsewhere[4] that rhodoquinone-9 is in fact a metabolite of ubiquinone-9. Whether or not rhodoquinone has a similar role in mitochondrial electron transport to that of ubiquinone is not yet known. Moore and Folkers[2] have suggested that, in the photosynthetic bacterium *R. rubrum*, rhodoquinone-10 may have a function in photosynthetic electron transport. The work reported here makes it clear that this is not its function in all photosynthetic micro-organisms.

Although not directly relevant to the main theme of this paper, another point in Table 4 is of interest. This is that the two carotenoid precursors, phytoene and phytofluene, appear to accumulate in the etiolated cells. This is contrary to the findings of Goodwin and Jamikorn[13] with different strains of *E. gracilis*. It appears that in etiolated *E. gracilis* var. *bacillaris* there is no marked reduction in the biosynthesis of phytoene and phytofluene but that the rate of their further metabolism to carotenoids is reduced. This leads to accumulation of the two compounds in etiolated cells. A detailed search for lycopersene was unsuccessful. This is in keeping with the idea that phytoene is the first hydrocarbon produced during the biosynthesis of carotenoids (see e.g. Ref. 14).

Other Organisms

The lipids of *E. gracilis* "strain Z" (No. 1224/7-4 of the Culture Collection of Algae and Protozoa, Botany School, Cambridge University) and of the blue-green alga *Anabaena* variabilis (made available through the kind cooperation of Dr. N. G. Carr) were also examined. The experiments on *Anabaena* were done concurrently with those reported recently by Carr and Hallaway.[15]

Rhodoquinone was present in the lipids of *E. gracilis* "strain Z" but none was detected in the lipids of *Anabaena*. This was not very surprising for, despite a thorough search, ubiquinone was also found to be absent.

[10] D. R. Threlfall and T. W. Goodwin, *Biochem. J.* **90**, 40 (1964).
[11] R. A. Dilley and F. L. Crane, *Plant Physiol.* **39**, 34 (1964).
[12] R. M. Smillie, *Can. J. Botany* **41**, 123 (1963).
[13] T. W. Goodwin and M. Jamikorn, *J. Protozool.* **6**, 216 (1954).
[14] B. H. Davies, D. Jones and T. W. Goodwin, *Biochem. J.* **87**, 326 (1963).
[15] N. G. Carr and H. M. Hallaway, *Biochem. J.* **97**, 9c (1965).

The further analysis of the lipids of *Anabaena* provided additional interest. The presence of plastoquinone-9, phylloquinone and α-tocopherol quinone was confirmed by u.v. spectroscopy and by co-chromatography with authentic compounds on thin layers of silica gel G and on paraffin-impregnated paper. As in *Euglena*, there was no evidence of the presence of plastoquinones-B, C or D. The levels of those quinones present were: phylloquinone 74 μg/g dry wt. (mean of two results, 73 and 75), plastoquinone-9 207 μg/g dry wt. (mean of three results, range 194–219) and α-tocopherol quinone 259 μg/g dry wt. (mean of three results, range 240–286). When the organism was grown with reduced light intensity, the levels of plastoquinone-9 (150 μg/g dry wt.) and of α-tocopherol quinone (167 μg/g dry wt.) were lower but the level of phylloquinone (74·5 μg/g dry wt.) was essentially the same as before. This may be indicative of a role for phylloquinone in non-photosynthetic electron transport in this particular organism.

α-Tocopherol also appeared to be missing from the lipid of *Anabaena*. Certainly the amount present must have been low. However, it was difficult to rule out the possibility of the presence of trace amounts, because of the presence of interfering carotenoids.

The evidence of the *Euglena* experiments was that ubiquinone and rhodoquinone are not associated with chloroplasts. They are most likely mitochondrial constituents. It is of taxonomic and evolutionary interest that in the procaryotic, photosynthetic micro-organism *Anaebaena* neither ubiquinone nor rhodoquinone were detected, whereas the normal plastidic quinones were present in relative abundance. It is of similar interest that in another procaryotic, photosynthetic micro-organism *R. rubrum* the reverse situation holds. The lipid of *R. rubrum* yields both mitochondrial quinones ubiquinone and rhodoquinone but does not contain the plastidic quinones plastoquinone[5] and phylloquinone (or menaquinone).[5] Investigations regarding the presence of tocopherol quinones in this organism have not yet been reported.

EXPERIMENTAL

Large-scale Isolation of Rhodoquinone-9 from Heterotrophic Cultures

The culture of *E. gracilis* var. *bacillaris* (culture 1224/7 from The Culture Collection of Algae and Protozoa, Botany School, Cambridge University) was grown initially at 28° in continuous light on slopes of composition 2·5 g NaCl, 3 g sodium acetate, 10 g peptone and 20 g agar, all made up to 1 l. with water. This was followed by subculturing a number of times in complete darkness. By this time the organism appeared to be entirely lacking in chlorophyll. The organism was then grown in 2-l. flasks containing 1 l. of the defined organic medium of Bach supplemented with vitamin B_{12} and metal ions.[16] Growth continued for 5 days at 28° in the dark either in a New Brunswick incubator with vigorous shaking or in an incubator oven with forced aeration. Altogether 40 l. culture was grown and this was harvested by centrifugation at 1600 *g* for 5 min and the packed cells were washed by resuspension in water followed by further centrifugation. The washed cells yielded approximately 100 g freeze-dried alga. This was then extracted at room temperature with 5% ethanol–ether (v/v) until all the lipid-soluble pigments were extracted. The ethanol was removed by washing twice with a solution of $(NH_4)_2SO_4$ (5% w/v), and the ethereal solution dried over Na_2SO_4 and evaporated finally under nitrogen. The resulting lipid (~27 g) was dissolved in light petroleum and chromatographed on 4 × 200 g columns of silicic acid–celite 545 (2:1). With each column, after running 1 l. 8% E–P (8% v/v diethyl ether in light

[16] M. K. BACH, *J. Protozool.* 7, 50 (1960).

petroleum), 1 l. 12% E–P eluted material containing rhodoquinone. This fraction was chromatographed preparatively on thin layers (250 μ thick) of silica gel G, using 30% (v/v) isopropyl ether in light petroleum as solvent. The rhodoquinons (the only purple band present) was extracted from the adsorbent with ether and, after removing the solvent, it was then crystallized a number of times from ethanol at −20° to yield 28 mg pure rhodoquinone-9 m.p. 66·5–67° (Kofler block).

Growth of Autotrophic Cultures of E. gracilis

E. gracilis was grown autotrophically in the chemically defined medium of Cramer and Myers,[17] 750 ml medium was used in 2-l. penicillin pots which were continuously gassed with 5% CO_2–air and incubated at 28° with adequate shaking and illumination (400 lx) from strip lights situated above the pots. The cells were harvested in the logarithmic phase after 5–7 days' growth, by centrifugation and the lipid was extracted as described above.

Unsaponifiable Constituents of Light- and Dark-grown Euglena

A number of analyses were made on the two types of cultures. On each occasion the lipid was chromatographed on acid-washed alumina of activity slightly greater than that of Brockmann Grade 3. Through each 10 g alumina was passed successively 100 ml each of light petroleum, 1% E–P, 4% E–P, 8% E–P, 15% E–P and 25% E–P.

Phytoene and phytofluene (when present) were eluted mainly by light petroleum but a little phytofluene appeared in the 1% E–P fraction. These compounds were recognized and assayed by the characteristic u.v. absorption showing, in cyclohexane, sharp λ_{max} at 276, 296 and 298 nm and at 333, 349 and 364 nm respectively (see e.g. Ref. 18). They were not present in the lipids of autotrophic *Euglena*. Thin-layer chromatography (silica gel G, 250 μ thick, light petroleum as solvent), using synthetic lycopersene as marker (R_f 0·55) failed to reveal the presence of lycopersene.

β-Carotene and saturated long-chain esters were quite apparent in the 1% E–P fractions. β-Carotene had the same u.v. absorption and R_f on thin-layer chromatograms as authentic β-carotene. The esters were identified by i.r. spectroscopy. In the etiolated cells these esters accounted for about 50 per cent of the total lipid. The corresponding figure for autotrophic cells was about 25 per cent. The ester is probably mainly myristyl myristate (see Rosenberg[19]). Traces of phylloquinone were detected in this fraction from the etiolated cells. Ergosteryl esters, plastoquinone-9 and a little α-tocopherol were eluted by 4% E–P. The plastoquinone-9 could be isolated by preparative thin-layer chromatography (silica gel G, 500 μ thick, 3% E–P as solvent). The quinone was then assayed by the change in absorption at 255 nm on reducing an ethanolic solution with sodium borohydride ($A_{1\,cm}^{1\%}=200$).[20] The amount of plastoquinone-9 in heterotrophic cells was less than 1 μg/g dry wt. Only plastoquinone A (plastoquinone-9) was encountered. This had the same R_f (0·28) as synthetic plastoquinone-9 when chromatographed on paraffin-impregnated paper, using 1% (v/v) water in dimethylformamide as solvent. Later chromatographic fractions failed to reveal the presence of either plastoquinone-C or plastoquinone-D.

Ubiquinone-9 and most of the α-tocopherol was eluted by 8% E–P. Ubiquinone was

[17] M. Cramer and J. Myers, *Arkiv. Mikrobiol.* **17**, 384 (1952).
[18] F. W. Hemming, R. A. Morton and J. F. Pennock, *Proc. Roy. Soc.* **B158**, 291 (1963).
[19] A. Rosenberg, *Biochemistry* **2**, 1148.
[20] F. L. Crane, *Ciba Found. Symp. Quinones in Electron Transport* 36 (1961).

estimated by the drop in absorption at 275 nm ($\Delta^{1\%}_{1cm} = 154$)[20] on adding sodium borohydride to an ethanolic solution. That the compound was the isoprenologue containing nine isoprene residues was shown by reversed-phase partition paper chromatography. The ubiquinone was first purified by preparative thin-layer chromatography (silica gel G (500 μ thick) 20% (v/v) isopropyl ether in light petroleum) and was then crystallized once from ethanol. On paraffin-impregnated paper, using dimethylformamide as solvent, the R_f of the *Euglena* ubiquinone was found to be identical with that of synthetic ubiquinone-9 (R_f 0·44), confirming the finding of Threlfall and Goodwin.[10]

The tocopherol was identified as α-tocopherol by two-dimensional thin-layer chromatography according to the method of Pennock, Hemming and Kerr.[21] Similar treatment of a portion of the total lipid failed to reveal the presence of any tocotrienols or tocopherols other than α-tocopherol. The level of α-tocopherol was estimated by the method of Whittle and Pennock (Private communication). This involved location of the tocopherol on a two-dimensional chromatogram by spraying with fluorescein and viewing under u.v. The tocopherol was eluted and estimated by the Emmerie–Engel method as recommended by the Analytical Methods Committee.[22]

The 15% E–P fractions contained rhodoquinone, ergosterol and α-tocopherol quinone. α-Tocopherol quinone was estimated by the change in absorption at 262 nm on reduction of an ethanolic solution with $NaBH_4$ ($\Delta E^{1\%}_{1cm} = 414$).[23] It was found difficult to estimate rhodoquinone with any accuracy in the presence of α-tocopherol quinone and ergosterol. The level of rhodoquinone in the lipid was more readily assayed by first subjecting the appropriate chromatographic fraction to two-dimensional thin-layer chromatography. The adsorbent was silica gel G (500 μ thick) and the solvent in the first dimension was 30% (v/v) isopropyl ether in light petroleum and benzene in the second dimension. In this way rhodoquinone could be separated from most other compounds, including carotenoids, ergosterol, tocopherol quinone and (when present) ubiquinone. The rhodoquinone (the only purple area on the chromatogram) was eluted from the chromatogram and estimated spectrophotometrically. It was found unsatisfactory to assay rhodoquinone by reducing with sodium borohydride. Bubbling nitrogen through the reduced solution was inconvenient and tended to concentrate the solution due to evaporation of the ethanol. Without bubbling nitrogen through the solution, reduction was never complete and the spectrum was changing continuously. Rhodoquinone was assayed directly from the spectrum in ethanol using the equation $E_{283}\,\text{corr} = 3{\cdot}78\,E_{283} - 1{\cdot}96\,E_{295} - 1{\cdot}84\,E_{269}$ to eliminate irrelevant absorption. This equation was derived from the spectrum of pure rhodoquinone-9, assuming that the irrelevant absorption was linear between 269 and 295 nm (cf. method of Morton and Stubbs[24] for vitamin A). The $E^{1\%}_{1cm}$ of pure rhodoquinone-9 was taken as 140.

Culture of Rhodospirillum rubrum

R. rubrum (NCIB 8255) was grown anaerobically at 30° in the light using the medium described by Goodwin and Osman.[25] Rhodoquinone was isolated from the cells in essentially the same manner as for *E. gracilis* and was used for comparisons throughout the investigations on the *Euglena* rhodoquinone.

[21] J. F. PENNOCK, F. W. HEMMING and J. D. KERR, *Biochem. Biophys. Res. Commun.* **17**, 542 (1964).
[22] Analytical Methods Committee, *Analyst* **84**, 356 (1959).
[23] C. BUCKE, Ph.D. Thesis, University of Liverpool (1965).
[24] R. A. MORTON and A. L. STUBBS, *Analyst* **71**, 348 (1965).
[25] T. W. GOODWIN and H. G. OSMAN, *Biochem. J.* **53**, 541 (1953).

Culture of Anabaena variabilis

A. variabilis was grown, in conjunction with Dr. N. G. Carr, on the autotrophic medium C described by Kratz and Myers,[26] supplemented with 0·5 g $NaHCO_3$/l., 0·004 g $Fe_2(SO_4)_3 . 6H_2O$/l. and 1 ml A5 micro-elements solution/l. The organism was cultured at 34° in penicillin pots, gassed with 5% CO_2–air with gentle shaking and illumination. The cells (0·49 g dry wt.) were harvested after 9–10 days in the late log. phase. The lipid was analysed in essentially the same way as was that from *Euglena*. Phylloquinone was present mainly in the 1% E–P fraction. It was separated from the other components by preparative thin-layer chromatography (silica gel G with 3% E–P as solvent). The u.v. absorption spectrum of the purified sample showed peaks in the same position as authentic phylloquinone and the two preparations had identical R_fs (0·57) on paraffin-impregnated paper using dimethylformamide as solvent.

Ammonolysis of Ubiquinone-9 and Ubiquinone-10

Moore and Folkers[2] report (without details) the ammonolysis of ubiquinone-10 to rhodoquinone-10. The present authors treated, at room temperature, solutions of ubiquinone-9 and ubiquinone-10 in ether–ethanol (1 : 1 v/v) either by stirring with 0·890 ammonia or by bubbling gaseous ammonia. Time intervals of between 2 and 160 hr were employed but on each occasion the yield of rhodoquinone was less than 1 per cent. The rhodoquinone was accompanied by unchanged ubiquinone and by a number of other reaction products some of which were also red. The synthetic rhodoquinones were separated from these by preparative thin-layer chromatography, using natural rhodoquinones as markers.

Spectrophotometry

Ultraviolet spectra were recorded with a Unicam SP-800 recording spectrophotometer. Extinction values at 520 nm involved in the quantitative Emmerie–Engel estimations were observed using a Unicam SP-600 spectrophotometer. Infrared absorption spectra were recorded with a Perkin–Elmer Infracord model 237.

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[26] W. A. Kratz and J. Myers, *Am. J. Botany* **42**, 282 (1955).