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THE BIOSYNTHESIS OF QUINONES FROM *p*-HYDROXYBENZOIC ACID IN *EUGLENA GRACILIS* VAR. *BACILLARIS**

R. POWLS† and F. W. HEMMING

Department of Biochemistry, University of Liverpool, Liverpool

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Abstract—Etiolated cultures of *E. gracilis* readily incorporated *p*-[U-^{14}C]hydroxybenzoic acid into ubiquinone-9. The evidence suggests that the ubiquinone-9 was then metabolized to rhodoquinone-9. Autotrophic cultures failed to incorporate *p*-[U-^{14}C]hydroxybenzoic acid into plastoquinone and α-tocopherol quinone although both ubiquinone and rhodoquinone were again labelled. Experiments with both sonicated and whole chloroplasts gave similar negative incorporation into the plastidic quinones. Whether this was the result of the chloroplast membrane being impermeable to *p*-hydroxybenzoic acid or due to this compound not being on the biosynthetic pathway of plastidic benzoquinones was not resolved.

INTRODUCTION

GLOVER and Threlfall[1] isolated a quinone, rhodoquinone, from *Rhodospirillum rubrum*, which appeared to be a desmethylubiquinone and they suggested that it might be the penultimate precursor of ubiquinone *in vivo*.

Parson and Rudney[2] showed that *p*-hydroxybenzoic acid was a very specific precursor of ubiquinone in *R. rubrum*, and moreover that ubiquinone acted as a precursor of rhodoquinone-10 in this organism. With the finding of rhodoquinone-9 in *Euglena gracilis*,[3] it was decided to investigate the biosynthetic interrelationship of the two quinones in this organism, especially as Moore and Folkers[4] had recently shown that rhodoquinone was an aminoquinone.

Autotrophic *E. gracilis*, in contrast to the heterotrophic organism, forms plastoquinone,[5] which in view of its similarity to ubiquinone might also be expected to be biosynthesized via *p*-hydroxybenzoic acid. The present paper describes studies on the biosynthesis of rhodoquinone, ubiquinone and plastoquinone in *E. gracilis*.

RESULTS AND DISCUSSION

Quinone Biosynthesis in Etiolated Heterotrophic Cultures of E. gracilis

Short-term incorporation of *p*-[U^{14}-C]hydroxybenzoic acid (PHBA) (1–6 hr) showed that activity passed from PHBA rapidly into ubiquinone whereas rhodoquinone became only

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† In receipt of a research studentship from the Department of Scientific and Industrial Research. Present address: Department of Biochemistry, University of Leicester.

[1] J. GLOVER and D. R. THRELFALL, *Biochem. J.* **85**, 14P (1962).
[2] W. W. PARSON and H. RUDNEY, *J. Biol. Chem.* **240**, 1855 (1965).
[3] R. POWLS and F. W. HEMMING, *Phytochem.* **5**, 1235 (1966).
[4] H. W. MOORE and K. FOLKERS, *J. Am. Chem. Soc.* **87**, 1409 (1965).
[5] R. C. FULLER, R. M. SMILLIE, N. RIGOPOULOS and V. YOUNT, *Arch. Biochem. Biophys.* **95**, 197 (1961).

slightly labelled (Fig. 1). This suggested that rhodoquinone was unlikely to be acting as a precursor of ubiquinone. In view of the weak labelling of rhodoquinone after 6 hr, longer-term incubations were thought to be necessary.

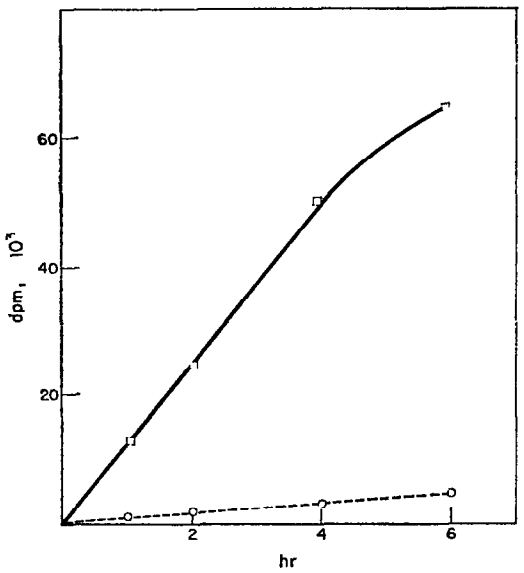

FIG. 1. THE CHANGE IN INCORPORATION OF RADIOACTIVITY INTO UBIQUINONE (—□—□—) AND RHODOQUINONE (—⊙—⊙—) WITH TIME AFTER INOCULATING A 3-DAY-OLD, ETIOLATED CULTURE OF *Euglena* WITH *p*-[U-^{14}C]HYDROXYBENZOATE (0·71 MG, $1{\cdot}32 \times 10^6$ DIS/MIN).

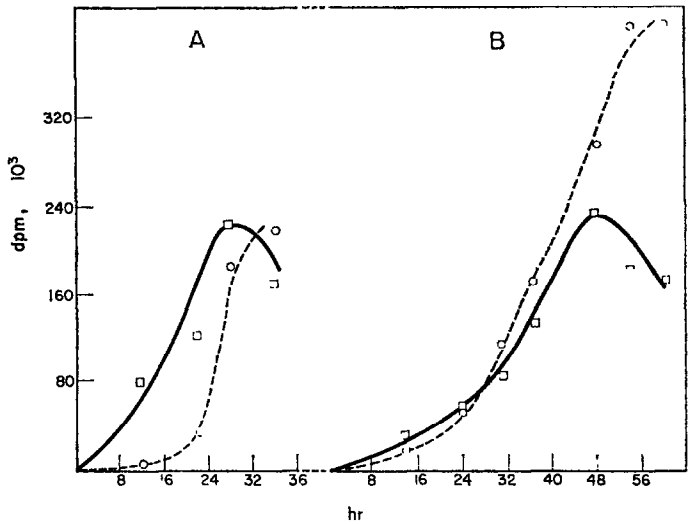

FIG. 2. THE CHANGE IN INCORPORATION OF RADIOACTIVITY INTO UBIQUINONE (—□—□—) AND RHODOQUINONE (—⊙—⊙—) WITH TIME AFTER INOCULATING (A) A 38-HR-OLD, ETIOLATED CULTURE AND (B) A 15-HR-OLD, ETIOLATED CULTURE OF *Euglena* WITH *p*-[U-^{14}C]HYDROXYBENZOATE (0·71 MG, $1{\cdot}32 \times 10^6$ DIS/MIN).

In a second experiment [U-^{14}C]PHBA was added to an etiolated culture after 38 hr growth in the dark. As growth in the dark continued, incorporation of radioactivity into ubiquinone rose rapidly to reach a peak about 28 hr (A, Fig. 2) after adding the PHBA. After ~24 hr, incorporation into rhodoquinone rose rapidly until, after 32 hr metabolism of PHBA, this quinone contained more ^{14}C than did ubiquinone. By this time the amount of ^{14}C in ubiquinone was falling.

In two further experiments it became clear that while the total radioactivity associated with rhodoquinone continued to rise well above the peak figure for ubiquinone, the specific activity of the rhodoquinone formed rose to a figure just a little lower than the maximum figure for ubiquinone. The results of one of these experiments are given in B, Fig. 2, and in B, Fig. 3. In this latter case [U-^{14}C]PHBA was added to a younger culture and it took almost twice as long for the incorporation of radioactivity into ubiquinone to reach a peak as in

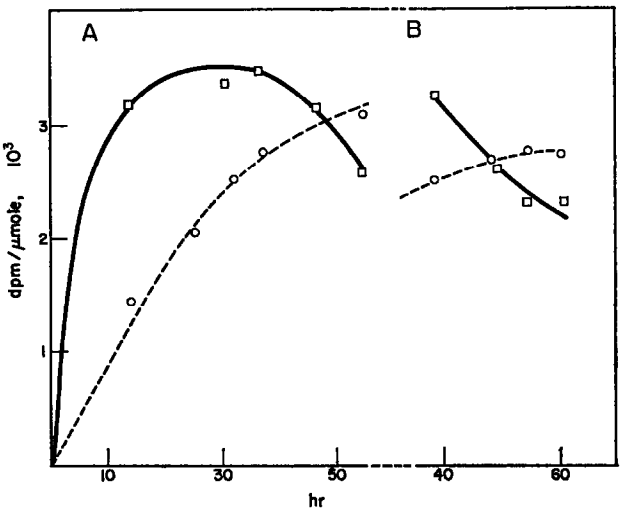

FIG. 3. THE CHANGE IN SPECIFIC ACTIVITY OF UBIQUINONE (—□—□—) AND RHODOQUINONE (—⊙—⊙—) WITH TIME AFTER INOCULATING A 15-HR-OLD, ETIOLATED CULTURE OF *Euglena* WITH *p*-[U-^{14}C] HYDROXYBENZOATE (0·71 MG, 1·32 × 10^6 DIS/MIN).

A and B are different experiments.

the previous experiment (Fig. 2A). The transfer of activity from ubiquinone to rhodoquinone again became rapid after ~24 hr. Consequently in this experiment, after about ~28 hr, labelling accumulated in rhodoquinone more rapidly than in ubiquinone. Presumably ubiquinone is converted to rhodoquinone more rapidly than rhodoquinone is metabolized. The specific activities of the two quinones were determined in the later stages of this experiment and are plotted in Fig. 3B. Also in Fig. 3A are plotted the specific activities of the quinones in a repeat experiment. It is clear from Fig. 3 that the specific activity of rhodoquinone reached a flat peak after ~54 hr. This peak value was only a little lower than the maximal value for ubiquinone.

Thus the incorporation of [U-^{14}C]PHBA into the quinones of etiolated *E. gracilis* follows the same pattern as in *R. rubrum*.[2] The evidence strongly suggests that, in *Euglena*, rhodoquinone is formed from ubiquinone, that it is probably the major metabolite of ubiquinone and that it is metabolized at a slower rate than is ubiquinone.

Radioautography of some of the thin-layer chromatograms was employed to check the possible presence of other radioactive metabolites of PHBA. There was no evidence of the presence of labelled 2-nonoprenyl phenol (cf. Ref. 6) or related compounds which might be precursors of ubiquinone-9, radioactivity being found only in ubiquinone and rhodoquinone.

Quinone Biosynthesis in Autotrophic Cultures of E. gracilis

Chromatography on alumina of the lipid obtained from autotrophic cultures of *E. gracilis* grown for 3 days in the presence of [U-^{14}C]PHBA (17·8 mg, $3{\cdot}31 \times 10^6$ dis/min) yielded most of the recovered radioactivity in the ubiquinone-containing fraction. The chromatographic fractions contained a total of 345 μg plastoquinone, 508 μg ubiquinone and 580 μg α-tocopherol quinone. Rhodoquinone was not assayed. Carrier plastoquinone-9, ubiquinone-9 and α-tocopherol quinone were added to the relevant fractions and purification continued, using preparative thin-layer chromatography. Both plastoquinone and α-tocopherol quinone were free of radioactivity. However, ^{14}C remained associated with the ubiquinone and after crystallization from ethanol to constant specific activity it could be calculated that the original endogenous ubiquinone had contained 23,590 dis/min. Rhodoquinone was only slightly labelled (172 dis/min) but since this experiment was done before a suitable technique for isolating rhodoquinone had been developed, it is most likely that only a portion of the rhodoquinone was recovered.

These results indicate that either PHBA is not a precursor of plastoquinone and α-tocopherol quinone in *E. gracilis* or, alternatively, that permeability factors were preventing its reaching the site of synthesis of these two quinones. This latter possibility is attractive since plastoquinone and α-tocopherol quinone occur in the chloroplasts whereas ubiquinone is a mitochondrial constituent.[7] Also, the chloroplast membrane has been shown to be relatively impermeable to a number of compounds, for example mevalonic acid.[8] However, it is important to note that, when exogenous [2-^{14}C]mevalonic acid is metabolized by plant tissues, the labelling recovered in plastoquinone and β-carotene has been reported to reach 30 and 36 per cent respectively of that incorporated into ubiquinone.[9] In *Euglena* no labelling at all was transferred from PHBA to plastoquinone. It would appear that, if the impermeability of the chloroplast membrane to PHBA is responsible for its lack of incorporation into plastoquinone, it is particularly effective. It is much more effective, for instance, than it is in preventing the incorporation of mevalonic acid into plastoquinone in higher plants.

Experiments with Isolated Chloroplasts

In an attempt to overcome permeability barriers attributable to the chloroplast membrane, studies were carried out with isolated chloroplasts. The chloroplasts were isolated using a non-aqueous technique and the membranes of a portion of these chloroplasts were ruptured by sonication. A suspension of broken chloroplasts containing added cofactors was incubated for 6 hr with [U-^{14}C]PHBA ($1{\cdot}32 \times 10^6$ dis/min). Carrier plastoquinone was added to the extracted lipid and after column and thin-layer chromatography followed by repeated crystallization from ethanol the final sample of pure plastoquinone was entirely free of ^{14}C. In a similar way it was also established that ^{14}C was not incorporated into α-tocopherol quinone.

[6] R. K. OLSEN, J. L. SMITH, K. FOLKERS, W. W. PARSON and H. RUDNEY, *J. Am. Chem. Soc.* **87**, 2298 (1965).
[7] D. R. THRELFALL and T. W. GOODWIN, *Biochem. J.* **90**, 40P (1964).
[8] T. W. GOODWIN and E. I. MERCER, *Biochem. Soc. Symp. (Cambridge, Engl.)*, No. 24, 37.
[9] D. R. THRELFALL, W. T. GRIFFITHS and T. W. GOODWIN, *Biochem. J.* **92**, 56 (1964).

When the portion of chloroplasts that was not sonicated was incubated with [U-^{14}C]-PHBA under the same conditions again, both plastoquinone and α-tocopherol quinone were recovered unlabelled.

General Conclusions

The experiments with etiolated cultures of *E. gracilis* provide good evidence that PHBA can be used as a precursor of ubiquinone-9 and that ubiquinone-9 is further metabolized to rhodoquinone-9. The accumulation of labelling in rhodoquinone-9 suggests that this quinone is metabolized less rapidly than is ubiquinone.

The results of experiments with autotrophic cultures of *Euglena* show quite clearly that PHBA is readily incorporated into ubiquinone which is situated outside the chloroplasts. The lack of incorporation of exogenous PHBA into the plastidic quinones, plastoquinone and α-tocopherol quinone, is equally clear. Although the results are strongly suggestive that PHBA is not a precursor of these plastidic quinones they do not exclude the possibility that the exogenous PHBA failed to permeate the chloroplast membrane. It is quite feasible that sonication of the chloroplast preparation resulted in loss of enzyme activity essential for the formation of plastidic benzoquinones.

EXPERIMENTAL

Quinone Biosynthesis in Etiolated, Heterotrophic Cultures

Euglena gracilis var. *bacillaris* (1224/7 Culture collection of algae and protozoa, Cambridge University) was grown in the dark at 28° with constant agitation in a New Brunswick orbital incubator. Batches of 1 l. of the defined medium of Bach[10] were grown in 2-l. flasks. When the culture was in the log phase of growth 0·4 ml of an ethanolic solution of [U-^{14}C]-PHBA (0·71 mg, $1{\cdot}32 \times 10^6$ dis/min) was added under sterile conditions. After the required time of incubation a portion of the culture was harvested by centrifugation and was frozen (−20°) immediately. The frozen material was lyophilized and extracted at room temperature with 5% ethanol–ether (v/v) until all the lipid was extracted. Washing with 5% (w/v) aq. ammonium sulphate removed the ethanol from the extract. After drying over Na_2SO_4 the ethereal extract was taken to dryness under nitrogen. The lipid was dissolved in petroleum (b.p. 40–60°) and chromatographed on a 10 g column of silicic acid–Celite 545 (2:1). The material eluted with 100 ml of 5% ether–petroleum (5% E–P) was discarded, the material eluted with 15% E–P, which on evaporation gave a red oil containing both ubiquinone and rhodoquinone, was dissolved in a small volume of cyclohexane. An aliquot of this solution containing 5–8 mg of lipid was applied as a spot to a silica gel G chromatoplate of 500 μ thickness which was developed in the first dimension with 30% di-isopropyl ether–petrol (v/v) followed by benzene in the second dimension. Rhodoquinone was easily visible as a purple spot and the ubiquinone could usually be visualized as a pale yellow spot (Fig. 4). Occasionally it was necessary to spray the plate with a 0·01% solution of sodium fluorescein in ethanol and then to detect the quinone in u.v. light.[11] The relevant areas of absorbent were scraped off from the plates, the quinones were eluted with ether and their specific activities were then determined.

[10] M. K. Bach, *J. Protozool.* 7, 50 (1960).
[11] P. J. Dunphy, K. J. Whittle and J. P. Pennock, *Chem. & Ind. (London)* 1217 (1965).

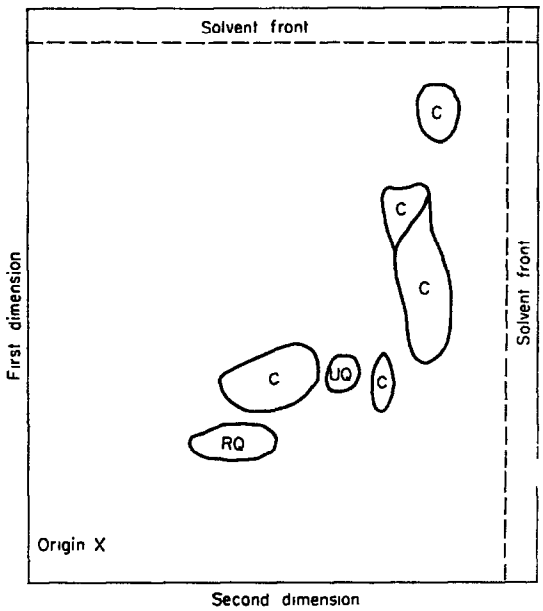

FIG. 4. TRACING OF A TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAM OF 5 MG OF THE 15% E–P FRACTION OF THE LIPID FROM ETIOLATED *Euglena* (SEE TEXT FOR DETAILS).

No staining—only naturally pigmented areas are shown. 1st dimension, 30% di-isopropyl ether–petrol; 2nd, benzene. C—carotenoid(s), UQ—ubiquinone, RQ—rhodoquinone.

Quinone Biosynthesis in Autotrophic Cultures

Autotrophic growth of *E. gracilis* was achieved in the medium of Cramer and Myers.[12] The organism was grown in 10 × 500 ml batches in 2 l.-penicillin pots which were maintained at 28° with continuous agitation, illumination of 50 lx and which were gassed with 5% CO_2–air. After 2 days' growth [U-^{14}C]PHBA (17·8 mg, $3{\cdot}31 \times 10^7$ dis/min) was added to the pots under sterile conditions. After a further 3 days' growth the cells were harvested and the lipid was extracted as previously described. The extracted lipid was chromatographed on an alumina column (20 g). Aliquots of fractions eluted by ether–petrol mixtures of increasing polarity were counted and their content determined by spectroscopy. Plastoquinone and ubiquinone were isolated from the relevant fractions by thin-layer chromatography on silica gel G after addition of carrier quinone. For the isolation of plastoquinone, 3% ether–petrol was used as developing solvent; for ubiquinone 1% methanol–benzene was used. The ubiquinone was crystallized from ethanol to constant specific activity after elution from the plates.

Quinone Biosynthesis in Isolated Chloroplasts

Chloroplasts of *E. gracilis* were isolated by the non-aqueous method of Smillie.[13] A portion of the chloroplast preparation was suspended in 5 ml of 0·2 M phosphate buffer, pH 7·4, containing the cofactors: 20 μM $MgSO_4$; 8 μM $MnCl_2$; 5 μM ATP; 120 μM tris; 13 μM ascorbic acid; 6 μM glutathione; 5 μM NADPH; 2×10^{-3} μM FMN.

[12] M. CRAMER and J. MYERS, *Arkiv. Mikrobiol.* **17**, 384 (1952).
[13] R. M. SMILLIE, *Can. J. Botany* **41**, 123 (1963).

This medium, with minor modifications, was adapted from that used by Heber and Tyskiewicz[14] for CO_2 fixation experiments with isolated chloroplasts. The homogenate so obtained was sonicated for 20 sec in an MSE 60 W ultrasonic disintegrater to rupture the chloroplast membranes. The sonicated extract was incubated with an aq. solution of sodium *p*-hydroxybenzoate ($1{\cdot}32 \times 10^6$ dis/min, prepared by neutralization of the free acid) for 6 hr at 27° with constant illumination and agitation. The reaction was terminated by the addition of acetone which also extracted the lipid. The extracted lipid was chromatographed on an alumina column, after addition of carrier plastoquinone. All the plastoquinone was eluted with 2% E–P and after purification on thin layers of silica gel G the quinone was crystallized from ethanol.

Determination of Specific Activities of Quinones

Radioactivity was determined with a Tricarb Liquid Scintillation System (Model 314 EX, Packard Instrument Co., U.S.A.). The scintillation solution was composed of 5 g 2,5-diphenyl oxazole (PPO) and 0·3 g of dimethyl-1-4-bis-2-(4-methyl-5-phenyloxazoyl)-benzene (dimethyl POPOP) in 1 l. toluene.

An aliquot of the ethereal quinone solution was transferred to a counting vial and the ether evaporated to dryness under nitrogen. Five ml of scintillation solution was added and the vial then counted. Correction for any quenching due to the quinones was made by using [^{14}C]toluene internal standards.

Quinones, in ethanolic solutions, were estimated by differences in their u.v. absorption when converted to the quinol by the addition of sodium borohydride. For ubiquinone-9 $\Delta E^{1\%}_{1\,cm,275} = 154$, for plastoquinone-9 $\Delta E^{1\%}_{1\,cm,255} = 200$.[15] The estimation of rhodoquinone-9 proved to be difficult. Ethanolic solutions of this quinone show selective absorption at 283 mμ; although reduction occurs with sodium borohydride, complex changes including reoxidation of the quinol rapidly occur in air. However if the reduction is carried out with nitrogen bubbling through the solution a typical quinol absorption spectrum is obtained, enabling the quinone to be estimated in the usual way.[2] In this work it was necessary to estimate very small quantities of rhodoquinone and this method proved impracticable. The only alternative was an estimation based upon the absorbance at 283 mμ of an ethanolic solution using the equation:

$$E_{283\,corr.} = 3{\cdot}78E_{283} - 1{\cdot}96E_{295} - 1{\cdot}84E_{269}$$

This equation eliminates irrelevant absorption and is derived from the spectrum of pure rhodoquinone-9 assuming any irrelevant absorption between 269 and 295 mμ to be linear. The $E^{1\%}_{1\,cm}$ of rhodoquinone used in the estimation was 146.

Acknowledgements—We should like to thank Dr. D. N. Burton for the generous gift of [U-^{14}C]PHBA prepared from [U-^{14}C]tyrosine by the method of Parson and Rudney.[16]

[14] U. Heber and E. Tyszkiewicz, *J. Exp. Bot.* **13**, 185 (1962).
[15] F. L. Crane, In *Ciba Found. Symp. Quinones Electron Transport*, 36 (1961).
[16] W. W. Parson and H. Rudney, *Proc. Natl Acad. Sci. U.S.* **51**, 444 (1964).