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Biosynthesis of Phytoquinones

HOMOGENTISIC ACID: A PRECURSOR OF PLASTOQUINONES, TOCOPHEROLS AND α-TOCOPHEROLQUINONE IN HIGHER PLANTS, GREEN ALGAE AND BLUE–GREEN ALGAE

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1. By means of ^{14}C tracer experiments and isotope competition experiments the roles of D-tyrosine, *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenylacetic acid, phenylacetic acid, homogentisic acid and homoarbutin (2-methylquinol 4-β-D-glucoside) in the biosynthesis of plastoquinones, tocopherols and α-tocopherolquinone by maize shoots was investigated. It was established that D-tyrosine, *p*-hydroxyphenylpyruvic acid and homogentisic acid can all be utilized for this purpose, whereas *p*-hydroxyphenylacetic acid, phenylacetic acid and homoarbutin cannot. Studies on the mode of incorporation of D-tyrosine, *p*-hydroxyphenylpyruvic acid and homogentisic acid showed that their nuclear carbon atoms and the side-chain carbon atom adjacent to the nucleus give rise (as a C_6-C_1 unit) to the *p*-benzoquinone rings and nuclear methyl groups (one in each case) of plastoquinone-9 and α-tocopherolquinone and the aromatic nuclei and nuclear methyl groups (one in each case) of γ-tocopherol and α-tocopherol. 2. By using [^{14}C]-homogentisic acid it has been shown that homogentisic acid is also a precursor of plastoquinone, tocopherols and α-tocopherolquinone in the higher plants *Lactuca sativa* and *Rumex sanguineus*, the green algae *Chlorella pyrenoidosa* and *Euglena gracilis* and the blue–green alga *Anacystis nidulans*.

It has been established that the aromatic carbon atoms and β-carbon atom of L-tyrosine can be utilized by maize shoots, French-bean shoots and ivy leaves for the biosynthesis of the nucleus and one nuclear methyl group respectively of each of the following compounds: plastoquinone-9, α-tocopherol, γ-tocopherol, δ-tocopherol (only shown in ivy) and α-tocopherolquinone (Whistance & Threlfall, 1967, 1968*a*). In maize shoots the remaining nuclear methyl groups and the polyprenyl side chains of plastoquinone-9, α-tocopherol, γ-tocopherol and α-tocopherolquinone are derived from the *S*-methyl group of methionine and mevalonic acid respectively (Threlfall, Whistance & Goodwin, 1968; Dada, Threlfall & Whistance, 1968).

To explain the incorporation of the β-carbon atom of tyrosine into plastoquinone-9, tocopherols and α-tocopherolquinone it has been postulated that one of the normal biosynthetic steps in their formation is an intramolecular rearrangement of *p*-hydroxyphenylpyruvic acid (Whistance & Threlfall, 1967, 1968*a*). This postulate, when considered in conjunction with the knowledge that shikimic acid is the distal precursor of the nuclei of plastoquinones, tocopherols and α-tocopherolquinone (Whistance, Threlfall & Goodwin, 1967) and with reports of the occurrence in plants of homogentisic acid (Bertel, 1903) and homoarbutin (2-methylquinol 4-β-D-glucoside) (Inoue, Arai & Takano, 1958), has led to the proposal that plastoquinones, tocopherols and tocopherolquinones might be synthesized by the following pathway: shikimic acid - - → *p*-hydroxyphenylpyruvic acid → homogentisic acid - - → homoarbutin - - → plastoquinones, tocopherols and tocopherolquinones (Whistance & Threlfall, 1967, 1968*a*). Some support for the belief that *p*-hydroxyphenylpyruvic acid and homogentisic acid are precursors of plastoquinone, tocopherol and tocopherolquinone has come from isotope competition experiments (Whistance & Threlfall, 1968*a*).

In the present paper we report further investigations into the roles of tyrosine, *p*-hydroxyphenylpyruvic acid, homogentisic acid and homoarbutin in the biosynthesis of plastoquinones, tocopherols and α-tocopherolquinone by higher plants, green algae and blue–green algae. Preliminary reports on some aspects of these studies have appeared already (Threlfall & Whistance, 1968; Whistance & Threlfall, 1968*b*).

EXPERIMENTAL

Radiochemicals

L-[U-^{14}C]Tyrosine hydrochloride (5.5mCi/mmol) and L-[U-^{14}C]phenylalanine (10mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. DL-[β-^{14}C]Tyrosine (*p*-hydroxyphenyl[3-^{14}C]alanine) (6.85mCi/mmol) was purchased from the New England Nuclear Corp., Boston, Mass., U.S.A.

Synthesis of p-*hydroxyphenyl*[3-^{14}C]*pyruvic acid and* D-[β-^{14}C]*tyrosine.* These were prepared by incubating DL-[β-^{14}C]tyrosine with L-amino acid oxidase [L-amino acid–oxygen oxidoreductase (deaminating), EC 1.4.3.2]. DL-[β-^{14}C]Tyrosine (50μCi; 6.85mCi/mmol) was dissolved in 0.5ml of 0.01M-HCl. A 1ml portion of 0.1M-potassium phosphate buffer, pH7.6, 10μl of catalase (hydrogen peroxide–hydrogen peroxide oxidoreductase, EC 1.11.1.6) [activity 3000 Bergmeyer (1955) units/100μl] and 2μl of L-amino acid oxidase [from *Crotalus adamanteus* venom, activity 1500 units (1 unit is the amount of enzyme required to deaminate 1μmol of L-leucine/min at 37°C and pH7.8)/100μl] were then added and the mixture incubated with gentle agitation for 30min at 37°C. At the end of this period the incubation mixture was transferred to a column (8cm × 0.8cm) of cation-exchange resin (Permutit Zeo-Karb 225). The column was developed with 10ml of 0.01M-HCl followed by 20ml of 2M-NH_3. The eluates were collected as 15 × 2ml fractions. *p*-Hydroxyphenyl-[3-^{14}C]pyruvic acid was eluted in fractions 1, 2 and 3; D-[β-^{14}C]tyrosine was eluted in fractions 9 and 10.

The radiochemical purities of the products were checked by descending paper chromatography [Whatman no. 1 paper with butan-1-ol–acetic acid–water (25:6:25, by vol.) as developing solvent] coupled with radioautography. It is of note that only 26% of the radioactivity in the DL-[β-^{14}C]tyrosine was associated with the L-isomer.

Synthesis of p-*hydroxy*[U-^{14}C]*phenylpyruvic acid and* [U-^{14}C]*phenylpyruvic acid.* These were synthesized from L-[U-^{14}C]tyrosine (20μCi; 5.5mCi/mmol) and L-[U-^{14}C]-phenylalanine (20μCi; 10mCi/mmol) respectively by the procedure described above.

Synthesis of p-*hydroxy*[U-^{14}C]*phenylacetic acid and* [U-^{14}C]*phenylacetic acid.* These were prepared by oxidative decarboxylation of *p*-hydroxy[U-^{14}C]phenylpyruvic acid and [U-^{14}C]phenylpyruvic acid respectively. The U-^{14}C-labelled keto acid (20μCi) was dissolved in 1ml of 0.001M-HCl and treated with 0.2ml of H_2O_2 ('10 volume'). After 30min the [U-^{14}C]phenylacetic acid was extracted from the reaction mixture with two 8ml portions of diethyl ether and chromatographed on a thin layer of Kieselgel G with benzene–methanol–acetic acid (45:8:4, by vol.) as developing solvent. (In this system *p*-hydroxyphenyl-acetic acid and phenylacetic acid have R_F values 0.6 and 0.7 respectively.) The yields of purified products were about 25%.

Synthesis of [U-^{14}C]*homogentisic acid and* [α-^{14}C]*homogentisic acid.* These were synthesized by incubating *p*-hydroxy[U-^{14}C]phenylpyruvic acid and *p*-hydroxy-phenyl[3-^{14}C]pyruvic acid respectively with $\alpha\alpha'$-bipyridyl-inhibited rat liver homogenates. The procedure employed was similar to that described by La Du & Zannoni (1955).

A fresh rat liver (6g) was homogenized in a Potter–Elvehjem homogenizer with 12ml of ice-cold 0.2M-sodium phosphate buffer, pH6.5. The homogenate was then centrifuged in a Sorvall Superspeed RC-2B refrigerated centrifuge at 1000*g* for 10min, after which the clear supernatant was decanted into a chilled flask.

The incubation with *p*-hydroxy[^{14}C]phenylpyruvic acid was carried out in a Warburg manometer flask set up as follows: main compartment, 1.0ml of rat liver preparation and 0.5ml of 0.1M-sodium phosphate buffer, pH6.5, containing 8μmol of ascorbic acid and 2μmol of $\alpha\alpha'$-bipyridyl; side arm, 0.5ml of 0.1M-sodium phosphate buffer, pH7.0, containing 20μCi of either L-[U-^{14}C]-tyrosine hydrochloride (5.5mCi/mmol) or DL-[β-^{14}C]-tyrosine (4.46mCi/mmol), 10μl of catalase (activity 3000 Bergmeyer units/100μl) and L-amino acid oxidase (from *Crotalus adamanteus* venom; activity 1500 units/100μl). The flask was equilibrated with slow oscillation at 37°C for 10min, during which time the [^{14}C]tyrosine in the side arm was converted into *p*-hydroxy[^{14}C]phenyl-pyruvic acid. The contents of the side arm were then added to the main compartment and the flask was shaken at 180 oscillations/min for 30min at 37°C.

At the end of the incubation period the contents of the flask were transferred to a 12ml stoppered test tube and, after acidification with 0.5ml of 3M-H_2SO_4, extracted twice with 8ml portions of ice-cold peroxide-free diethyl ether. The ethereal extracts were combined and concentrated to about 0.5ml by evaporation under a stream of O_2-free N_2. The extract was then chromatographed on a thin layer of Kieselgel G with benzene–methanol–acetic acid (45:8:4, by vol.) as developing solvent. The developed chromatogram was sprayed lightly with 0.2% (w/v) Rhodamine 6G in acetone, and the homogentisic acid, visible under u.v. light as a dark blue band (R_F 0.3), was recovered from the gel by extraction with diethyl ether or water. The yield of purified product was usually 85–90% of the theoretical.

The radiochemical purity of the acid was established as follows. A sample was oxidized with $FeCl_3$ to *p*-benzoquinoneacetic acid: this was then chromatographed on a thin layer of Kieselgel G with benzene–methanol–acetic acid (45:8:4, by vol.) as developing solvent. Radioautography of the developed plate showed that all the applied radioactivity had migrated with the same R_F (0.6) as authentic *p*-benzoquinoneacetic acid. [^{14}C]Homogentisic acid samples were stored at 4°C in 10ml of water containing 10mg of ascorbic acid. The latter is required to keep the homogentisic acid in its reduced form.

Chemicals

Isolation of homoarbutin from Pyrola rotundifolia. Leaves (100g wet wt.) of *P. rotundifolia* var. *maritima* (collected from the dune slacks at Formby, Lancs., U.K., in July 1968) were refluxed for 1h with 1.5 litres of aq. 80% (v/v) ethanol. After filtration and removal of the solvent (by rotary evaporation), the water-soluble portion of the extract was dissolved in 500ml of boiling water and passed through a column (15cm × 2.5cm) of polyamide (120–500 mesh). The eluate, containing the homoarbutin, was collected and freeze-dried. The resulting light-brown residue was chromatographed on thin layers of Kieselgel G (prewashed with aq. 80% ethanol to remove binder) with ethyl acetate–butan-2-one–formic acid–water (5:3:1:1, by vol.) as developing solvent. The developed plates

were sprayed lightly with 0.2% Rhodamine 6G in acetone, and the homoarbutin, visible under u.v. light as a purple band (R_F 0.3), was recovered from the gel by extraction with aq. 90% (v/v) ethanol. This procedure gave 300mg of pure homoarbutin [m.p. 110°C (uncorr.); $\lambda_{max.}$ 284nm in aq. 90% (v/v) ethanol].

Other chemicals. The sources and grades of the other chemicals used were as given by Whistance & Threlfall (1968*a*).

Biological methods

Biological material. Seeds of *Zea mays* var. Rhodesian Double White Hybrid were purchased from Hurst, Gunson, Cooper and Taber Ltd., Witham, Essex, U.K. Leaves of *Lactuca sativa* (lettuce) and *Rumex sanguineus* (red-veined dock) were obtained locally in June 1969. Cultures of *Euglena gracilis* strain Z and *Chlorella pyrenoidosa* 211-8H were purchased from the Culture Collection of Algae and Protozoa, Cambridge, U.K. The culture of *Anacystis nidulans* (M. B. Allen) was a gift from Dr A. J. Smith of this Department.

Cultivation of maize seedlings. Etiolated 6–7-day-old maize seedlings were grown in the manner described by Griffiths, Threlfall & Goodwin (1967).

Culture of algae. (*a*) *E. gracilis* strain Z was grown under the conditions described by Whistance & Threlfall (1968*b*). (*b*) *A. nidulans* was grown in the manner described by Smith, London & Stanier (1967). (*c*) *C. pyrenoidosa* was grown in static culture on the medium described by Goodwin (1954). The cells were grown for 5 days in the light (300lm/ft^2) at 28°C in 2-litre flasks, each containing 1 litre of medium.

Exposure of biological material to ^{14}C-labelled substrates. Etiolated maize shoots were excised at the node and the cut ends were dipped in 0.1M-potassium phosphate buffer, pH7.0 (50ml/100 shoots), containing the radioactive substrate. They were then exposed to continuous illumination (300lm/ft^2) for 24h (16h in isotope competition experiments) at 28°C. Dock leaves were incubated in a similar manner, except the incubation period was of only 6h duration.

In the experiment with lettuce, the inner leaves of four lettuce heads were excised and passed through 0.1M-potassium phosphate buffer, pH7.0 (25ml), containing the radioactive substrate and 0.1% of Tween 80. They were then spread on a large glass plate and illuminated (300lm/ft^2) for 6h at 28°C.

C. pyrenoidosa, *A. nidulans* and *E. gracilis* were harvested by centrifugation, resuspended in 200ml of 0.05M-potassium phosphate buffer, pH7.0, containing the radioactive substrate and exposed with shaking to continuous illumination (300lm/ft^2) for 4h at 28°C.

[^{14}C]Homogentisic acid was administered in the presence of ascorbic acid (50mg/100ml of incubation medium). This was necessary in order to keep it reduced.

Analytical methods

Extraction and preliminary fractionation of lipid. Higher-plant tissues were extracted by the procedure of Griffiths *et al.* (1967). Cells of *E. gracilis*, *C. pyrenoidosa* and *A. nidulans* were extracted by the method of Threlfall & Goodwin (1967).

The lipids were resolved into various isoprenoid quinone- and chromanol-containing fractions by chromatography on columns of acid-washed alumina (Brockmann grade III) (Woelm; anionotropic) developed by stepwise elution with 0.25, 1, 3, 5, 8, 12 and 20% E/P* as described by Griffiths *et al.* (1967).

Purification procedures. The purifications of phylloquinone, plastoquinone-9, plastoquinone-8, plastoquinone B, phytylplastoquinone, phytylplastohydroquinone monomethyl ether, ubiquinone, α-tocopherolquinone, γ-tocopherol, α-tocopherol, β-carotene and 4-demethylsterols were carried out as described by Whistance *et al.* (1967) and Whistance & Threlfall (1968*a*, 1970*a*,*b*).

Rhodoquinone-9, present in the 12% E/P fraction obtained from chromatography of the *E. gracilis* lipid on a column of alumina, was purified by chromatography on thin layers of Kieselgel G developed with (i) chloroform (R_F 0.55), (ii) aq. 90% (v/v) acetone (reversed-phase) (R_F 0.41) and (iii) di-isopropyl ether–benzene (1:4, v/v) (R_F 0.5).

Hydroxyphylloquinone, present in the 12% and 20% E/P fractions obtained from chromatography of the *A. nidulans* and *C. pyrenoidosa* lipids on columns of alumina, was purified in a manner identical with that described for rhodoquinone. The R_F values in the various solvent systems were 0.4, 0.76 and 0.33 respectively.

Determination of quinones, chromanols, β-carotene and 4-demethylsterols. Phylloquinone, plastoquinone-9, plastoquinone B, phytylplastoquinone, phytylplastohydroquinone monomethyl ether, ubiquinone, α-tocopherolquinone, γ-tocopherol, α-tocopherol, β-carotene and 4-demethylsterols were assayed by the procedures of Threlfall & Goodwin (1967), Whistance *et al.* (1967) and Whistance & Threlfall (1968*a*, 1970*a*). Rhodoquinone was assayed in cyclohexane by using an $E^{1\%}_{1cm}$ (280nm) value of 140 (Powls & Hemming, 1966).

Chemical degradation of isoprenoid quinones and chromanols. Radioactivity in the polyprenyl side chains of plastoquinone and ubiquinone was determined by ozonolytic degradation (Whistance *et al.* 1967). Radioactivity in the nuclear methyl groups of plastoquinone was determined by a combination of Kuhn–Roth oxidation and a Schmidt degradation (Threlfall *et al.* 1968).

Spectrophotometry. All u.v. and visible spectra were determined in a Unicam SP. 800 spectrophotometer.

Radioassay. The methods employed have been described by Whistance & Threlfall (1968*a*). All counts were corrected for background and instrument efficiency.

RESULTS

Administration of radioactive D-*tyrosine,* p-*hydroxyphenylpyruvic acid,* p-*hydroxyphenylacetic acid and homogentisic acid to maize shoots.* Whistance & Threlfall (1968*a*) obtained results from isotope competition experiments which suggested that maize shoots can utilize *p*-hydroxyphenylpyruvic acid, homogentisic acid and D-tyrosine for the biosynthesis of plastoquinone-9, α-tocopherol,

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

Table 1. *Incorporation of* D-[β-^{14}C]*tyrosine*, p-*hydroxyphenyl*[3-^{14}C]*pyruvic acid*, p-*hydroxy*[U-^{14}C]-*phenylpyruvic acid*, [α-^{14}C]*homogentisic acid and* [U-^{14}C]*homogentisic acid into isoprenoid quinones and chromanols*, β-*carotene and* 4-*demethylsterols by maize shoots*

Etiolated 7-day-old maize shoots were excised and exposed with continuous illumination to the appropriate radioactive substrate for 24h. N.D., Not determined.

Terpenoids examined	Specific radioactivity (c.p.m./μmol)				
	D-[β-^{14}C]-Tyrosine (6.85mCi/mmol): 200 shoots incubated with 5μCi	p-Hydroxyphenyl-[3-^{14}C]pyruvic acid (6.85mCi/mmol): 200 shoots incubated with 5μCi	p-Hydroxy[U-^{14}C]-phenylpyruvic acid (5.5mCi/mmol): 200 shoots incubated with 5μCi	[α-^{14}C]-Homogentisic acid (6.85mCi/mmol): 200 shoots incubated with 3μCi	[U-^{14}C]-Homogentisic acid (4.9mCi/mmol): 200 shoots incubated with 3μCi
Intrachloroplastidic:					
β-Carotene	51	29	49	268	370
Phylloquinone	115	54	60	360	242
Plastoquinone-9	32150	4190	6915	71600	73800
Plastochromanol	0	0	0	0	0
γ-Tocopherol	39367	11830	19827	307500	294800
α-Tocopherol	8843	2900	3120	43200	35840
α-Tocopherolquinone	10700	3625	3400	46250	36460
Extrachloroplastidic:					
4-Demethylsterols	253	89	152	348	232
Ubiquinone-9	498	458	N.D.	1740	1384

Table 2. *Chemical degradation of plastoquinone-9 samples isolated from maize shoots exposed to* ^{14}C-*labelled species of* p-*hydroxyphenylpyruvic acid and homogentisic acid*

Substrate	Ozonolytic degradation			Kuhn–Roth oxidation			
		Radioactivity in nonaprenyl side chain			Radioactivity in sodium acetate		
	Amount degraded (c.p.m.)	(c.p.m.)	(% of total radioactivity in molecule)	Amount degraded (c.p.m.)	(% of total radioactivity in molecule)	(% of radioactivity in carboxyl group)	(% of radioactivity in methyl group)
[U-^{14}C]Homogentisic acid	10300	150	1.5	16690	45	67	33
[α-^{14}C]Homogentisic acid	25180	261	1.0	32140	97	0	100
p-Hydroxy[U-^{14}C]-phenylpyruvic acid	—	—	—	8309	41	63	30
p-Hydroxyphenyl-[3-^{14}C]pyruvic acid	—	—	—	6420	94	0	100

γ-tocopherol and α-tocopherolquinone. To investigate this further the incorporation of D-[β-^{14}C]-tyrosine, p-hydroxyphenyl[3-^{14}C]pyruvic acid, p-hydroxy[U-^{14}C]phenylpyruvic acid, [α-^{14}C]homogentisic acid and [U-^{14}C]homogentisic acid into these quinones and chromanols by maize shoots was examined (Table 1). In the same series of investigations experiments were carried out with p-hydroxy[U-^{14}C]phenylacetic acid (3μCi administered to 200 shoots) and [U-^{14}C]phenylacetic acid (3μCi administered to 200 shoots), compounds that on structural grounds can also be envisaged as precursors of plastoquinone-9, tocopherols and α-tocopherolquinone.

In the experiments with D-[β-^{14}C]tyrosine, p-hydroxy[^{14}C]phenylpyruvic acid and [^{14}C]homogentisic acid radioactivity was incorporated into all compounds examined (Table 1). In the experiments with p-hydroxy[U-^{14}C]phenylacetic acid and [U-^{14}C]phenylacetic acid, on the other hand, no incorporation of radioactivity took place. Appropriate chemical degradations of the plastoquinone-9

samples (Table 2), together with comparisons of the specific radioactivities of β-carotene, phylloquinone and 4-demethylsterols with those of the quinones and chromanols under investigation (Table 1), established that (*a*) the radioactivity incorporated into plastoquinone-9, α-tocopherol, γ-tocopherol and α-tocopherolquinone from D-[β-^{14}C]tyrosine, *p*-hydroxyphenyl[3-^{14}C]pyruvic acid and [α-^{14}C]-homogentisic acid was present almost entirely in nuclear methyl groups (one in each compound), (*b*) the radioactivity incorporated into plastoquinone-9, α-tocopherol, γ-tocopherol and α-tocopherolquinone from *p*-hydroxy[U-^{14}C]phenylpyruvic acid and [U-^{14}C]homogentisic acid was distributed uniformly amongst the nuclear carbon atoms and nuclear methyl groups (one in each compound) and (*c*) the radioactivity incorporated into ubiquinone from *p*-hydroxyphenyl[3-^{14}C]pyruvic acid, [^{14}C]homogentisic acid and D-[β-^{14}C]tyrosine (the ubiquinone labelled from *p*-hydroxy[U-^{14}C]phenylpyruvic acid was not isolated) was present entirely in the nonaprenyl side chain.

Administration of [U-^{14}C]*homogentisic acid to higher plants, green algae and blue–green algae.* To determine whether homogentisic acid is a precursor of plastoquinones, tocopherols and tocopherolquinones in higher plants (other than maize), green algae and blue–green algae a small survey was carried out in which [U-^{14}C]homogentisic acid was administered to *Lactuca sativa* (Table 3), *Rumex sanguineus* (Table 4), *Euglena gracilis* (Table 5), *Chlorella pyrenoidosa* (Table 6) and *Anacystis nidulans* (Table 7).

Radioactivity was incorporated into plastoquinone-9 by each of the six organisms. It was also incorporated into α-tocopherol in *L. sativa*, *R. sanguineus*, *E. gracilis* and *C. pyrenoidosa*, γ-tocopherol in *L. sativa* and *R. sanguineus*, α-tocopherolquinone in *L. sativa*, and plastoquinone-8, phytylplastoquinone and phytylplastohydroquinone

Table 3. *Incorporation of* [U-^{14}C]*homogentisic acid into isoprenoid quinones and chromanols by* Lactuca sativa

Leaves of *L. sativa* (230g wet wt.) were coated with 25ml of 0.05M-potassium phosphate buffer, pH 7.0, containing 1.5μCi of [U-^{14}C]homogentisic acid and 12mg of ascorbic acid, and incubated for 6h at room temperature with continuous illumination. The lipid (375mg) was then extracted and chromatographed on a column of Brockmann grade III acid-washed alumina.

Fraction (% E/P)	^{14}C Radioactivity (c.p.m.)	Terpenoids examined	Amount (μmol)	Specific radioactivity (c.p.m./μmol)
0.25	2000	β-Carotene	1.49	115
1	8060	Plastoquinone-9	2.09	4590
3	11160	α-Tocopherol	2.71	3740
		Plastoquinone-B	0.12	0
5	23750	γ-Tocopherol	1.44	12040
		Ubiquinone-10	0.30	812
12	9350	4-Demethylsterols	35.70	133
20	6830	α-Tocopherolquinone	0.28	5560

Table 4. *Incorporation of* [U-^{14}C]*homogentisic acid into isoprenoid quinones and chromanols by* Rumex sanguineus

Leaves of *R. sanguineus* (91g wet wt.) were dipped into 25ml of 0.05M-potassium phosphate buffer, pH 7.0, containing 1.5μCi of [U-^{14}C]homogentisic acid and 12mg of ascorbic acid, and incubated for 6h at room temperature with continuous illumination. The lipid (285mg) was then extracted and chromatographed on a column of Brockmann grade III acid-washed alumina.

Fraction (% E/P)	^{14}C Radioactivity (c.p.m.)	Terpenoids examined	Amount (μmol)	Specific radioactivity (c.p.m./μmol)
0.25	15800	β-Carotene	5.58	0
		Phylloquinone	1.75	0
1	37300	Plastoquinone-9	3.23	5010
3	45600	α-Tocopherol	11.64	2885
5	35710	γ-Tocopherol	0.23	27750
		Ubiquinone-9	0.50	2740*
12	40500	4-Demethylsterols	62.20	489
20	2560	—	—	—

* All the radioactivity was located in the nonaprenyl side chain.

Table 5. *Incorporation of* [U-^{14}C]*homogentisic acid into isoprenoid quinones and chromanols by* Euglena gracilis

Light-grown 7-day-old cells of *E. gracilis* strain Z (3.6g wet wt.) from 10 litres of culture medium were suspended in 200ml of 0.05M-potassium phosphate buffer, pH 7.0, containing 1.5 μCi of [U-^{14}C]homogentisic acid and 100mg of ascorbic acid, and exposed to continuous illumination for 4h with shaking at 28°C. The lipid (390mg) was then extracted and chromatographed on a column of Brockmann grade III acid-washed alumina.

Fraction (% E/P)	^{14}C Radioactivity (c.p.m.)	Terpenoids examined	Amount (μmol)	Specific radioactivity (c.p.m./μmol)
0.25	19540	β-Carotene	3.73	164
		Phylloquinone	0.03	87
		Phytylplastoquinone	0.20	3620
		Phytylplastohydroquinone monomethyl ether	0.93	981
1	30500	Plastoquinone-9	4.68	2420
		Plastoquinone-8	0.09	5220*
3	8240	α-Tocopherol	7.40	1930
5	1720	Ubiquinone-9	0.18	413
12	510	4-Demethylsterols	3.38	60
		Rhodoquinone-9	0.63	0
20	190	—	—	—

* Details of the discovery of this quinone are given by Whistance & Threlfall (1970*b*).

Table 6. *Incorporation of* [U-^{14}C]*homogentisic acid into isoprenoid quinones and chromanols by* Chlorella pyrenoidosa

Light-grown 5-day-old cells of *C. pyrenoidosa* (6g wet wt.) from 10 litres of culture medium were suspended in 200ml of 0.05M-potassium phosphate buffer, pH 7.0, containing 1.5 μCi of [U-^{14}C]homogentisic acid and 100mg of ascorbic acid, and exposed to continuous illumination for 4h with shaking at 28°C. The lipid (285mg) was then extracted and chromatographed on a column of Brockmann grade III acid-washed alumina.

Fraction (% E/P)	^{14}C Radioactivity (c.p.m.)	Terpenoids examined	Amount (μmol)	Specific radioactivity (c.p.m./μmol)
0.25	0	β-Carotene	0.57	0
		Phylloquinone	0.03	0
1	5920	Plastoquinone-9	1.87	3420
3	3360	α-Tocopherol	1.62	1990
5	6300	Ubiquinone-10	0.29	0
12	1308	4-Demethylsterols	5.80	40
		Hydroxyphylloquinone*	0.04	0
20	1540	Hydroxyphylloquinone*	0.04	0

* This quinone had u.v. and t.l.c. properties identical with those of the hydroxyphylloquinone isolated from *A. nidulans* (Table 7 and Allen, Franke & Hirayama, 1967).

monomethyl ether in *E. gracilis*. Appropriate chemical degradations of the plastoquinone-9 samples (Table 8), together with comparisons of the specific radioactivities of β-carotene, phylloquinone and 4-demethylsterols with those of the quinones and chromanols under investigation (Tables 3–7), established that in all cases the radioactivity present in the above compounds was distributed uniformly among the nuclear carbon atoms and nuclear methyl groups (one in each compound).

Isotope competition experiments. (*a*) Experiment with homogentisic acid. Whistance & Threlfall (1967, 1968*a*) proposed that the homogentisic acid utilized by higher plants for the biosynthesis of plastoquinones, tocopherols and tocopherolquinones arises directly from *p*-hydroxyphenylpyruvic acid, presumably by a reaction similar to that which takes place in animals and some micro-organisms. To investigate this the incorporation of radioactivity into plastoquinone-9, tocopherols and α-tocopherolquinone by greening etiolated maize shoots metabolizing 5 μCi of *p*-hydroxy[U-^{14}C]phenylpyruvic acid in the presence or absence of 500 μmol of homogentisic acid was examined.

Analysis of the tissues at the end of the incubations showed that the homogentisic acid had depressed the incorporation of radioactivity into

Table 7. *Incorporation of* [U-^{14}C]*homogentisic acid into isoprenoid quinones and chromanols by* Anacystis nidulans

Light-grown 5-day-old cells of *A. nidulans* (1.6g wet wt.) from 4 litres of culture medium were suspended in 200ml of 0.05M-potassium phosphate buffer, pH7.0, containing 1.5μCi of [U-^{14}C]homogentisic acid and 100mg of ascorbic acid, and exposed to continuous illumination for 4h at 28°C. The lipid (94mg) was then extracted and chromatographed on a column of Brockmann grade III acid-washed alumina.

Fraction (% E/P)	^{14}C Radioactivity (c.p.m.)	Terpenoids examined	Amount (μmol)	Specific radioactivity (c.p.m./μmol)
0.25	1003	β-Carotene	—	0
		Phylloquinone	0.14	0
1	7930	Plastoquinone-9	0.67	9260
3	1250	—	—	—
5	670	—	—	—
12	0	Hydroxyphylloquinone*	0.15	0
20	324	Hydroxyphylloquinone*	0.11	0

* This quinone has been isolated previously from *A. nidulans* by Allen *et al.* (1967).

Table 8. *Chemical degradations of plastoquinone samples isolated from* Lactuca sativa, Rumex sanguineus, Euglena gracilis, Chlorella pyrenoidosa *and* Anacystis nidulans *exposed to* [U-^{14}C]*homogentisic acid*

Source of sample	Ozonolytic degradation: Amount degraded (c.p.m.)	Radioactivity in nonaprenyl side chain (c.p.m.)	Radioactivity in nonaprenyl side chain (% of total radioactivity in molecule)	Kuhn–Roth oxidation: Amount degraded (c.p.m.)	Radioactivity in sodium acetate (% of total radioactivity in molecule)
L. sativa	4500	0	<2	4500	40
R. sanguineus	7300	0	<2	7300	41
E. gracilis	5010	0	<2	5010	39
C. pyrenoidosa	—	—	—	5820	39
A. nidulans	—	—	—	5750	44

each of the quinones and chromanols examined by more than 90%.

(*b*) Experiment with homoarbutin. Whistance & Threlfall (1967, 1968*a*) proposed homoarbutin as an intermediate in the conversion of homogentisic acid into plastoquinones, tocopherols and tocopherolquinones in higher plants. However, isotope competition experiments and ^{14}C tracer studies with its aglycone, toluquinol, failed to provide any evidence of such a role, although the possibility existed that the experimental tissues (maize and French-bean shoots) were unable to convert the administered toluquinol into homoarbutin (Whistance & Threlfall, 1968*a*). To clarify this situation the incorporation of radioactivity into plastoquinone-9, tocopherols and α-tocopherolquinone by greening etiolated maize shoots metabolizing 2μCi of [U-^{14}C]homogentisic acid in the presence or absence of 500μmol of homoarbutin was examined.

On analysis of the tissues at the end of the incubations it was found that the homoarbutin had not affected the incorporation of radioactivity into plastoquinone-9, α-tocopherol, γ-tocopherol or α-tocopherolquinone.

DISCUSSION

In keeping with the proposals of Whistance & Threlfall (1967, 1968*a*) *p*-hydroxyphenylpyruvic acid and homogentisic acid were found to be precursors of plastoquinone-9, α-tocopherol, γ-tocopherol and α-tocopherolquinone in maize shoots (Table 1). Homogentisic acid (*p*-hydroxyphenylpyruvic acid was not tested) was also found to be a precursor of plastoquinone, tocopherol and tocopherolquinone in the higher plants *L. sativa* (Table 3) and *R. sanguineus* (Table 4), the green algae *E. gracilis* (Table 5) and *C. pyrenoidosa* (Table 6) and the blue-green alga *A. nidulans*

(Table 7). Chemical degradations (Tables 2 and 8), together with comparisons of the specific radioactivities of β-carotene, phylloquinone and 4-demethylsterols with those of the compounds under investigation (Tables 1 and 3–7), established that the nuclear carbon atoms and α-carbon atom of homogentisic acid, in the form of a C_6–C_1 unit, give rise to the *p*-benzoquinone rings and nuclear methyl groups (one in each compound) of plastoquinone-9, plastoquinone-8, phytylplastoquinone, phytylplastohydroquinone monomethyl ether and α-tocopherolquinone, and the aromatic rings and nuclear methyl groups (one in each compound) of γ-tocopherol and α-tocopherol. By using similar methods it was shown that in maize shoots *p*-hydroxyphenylpyruvic acid also gives rise to a C_6-C_1 unit in plastoquinone-9, α-tocopherol, γ-tocopherol and α-tocopherolquinone (Tables 1 and 2).

There seems little doubt that in maize shoots homogentisic acid arises from *p*-hydroxyphenylpyruvic acid. Evidence for this are the facts that (*a*) radioactivity from [^{14}C]homogentisic acid is incorporated far more effectively than radioactivity from *p*-hydroxy[^{14}C]phenylpyruvic acid into plastoquinone-9, tocopherols and α-tocopherolquinone (Table 1), (*b*) homogentisic acid markedly diminishes the incorporation of radioactivity from *p*-hydroxy-[U-^{14}C]phenylpyruvic acid into plastoquinone-9, tocopherols and α-tocopherolquinone and (*c*) radioactivity from [U-^{14}C]phenylacetic acid was not incorporated into any of the quinones and chromanols examined (this excludes the possibility that homogentisic acid arises via *ortho*- and *meta*-hydroxylation of phenylacetic acid, as it does in some micro-organisms; Evans, 1963). The mechanism of the conversion remains to be defined. However, the finding that *p*-hydroxyphenylacetic acid (a compound that can be visualized as an intermediate in the conversion of *p*-hydroxyphenylpyruvic acid into homogentisic acid) is not involved in the biosynthesis of plastoquinone-9, tocopherol and tocopherolquinone (Table 1) suggests that, as proposed by Whistance & Threlfall (1967, 1968*a*), it is similar to that in animals (see, e.g., Meister, 1965) and some micro-organisms (Evans, 1963) i.e. hydroxylation of the aromatic ring, shift of the side chains and the formation of carbon dioxide.

Nothing is known about the intermediate steps in the synthesis of plastoquinones, tocopherols and α-tocopherolquinone from homogentisic acid, although it is clear that in maize shoots, at least, the C_6–C_1 compounds gentisic acid, gentisaldehyde, gentisyl alcohol, toluquinol (Whistance & Threlfall, 1968*a*) and homoarbutin (see the Results section) are not involved. In accordance with previous proposals (Whistance & Threlfall, 1967, 1968*a*) it is suggested that in plastoquinones, tocopherols, tocopherolquinones and all biosynthetically related compounds the α-carbon atom of homogentisic acid gives rise to the nuclear methyl group ***meta*** to the polyprenyl side chain.

During the investigation it was established unequivocally that maize shoots can utilize D-tyrosine for the biosynthesis of plastoquinone-9, γ-tocopherol, α-tocopherol and α-tocopherolquinone (Table 1). It seems likely that to be utilized for this purpose D-tyrosine must first be converted into *p*-hydroxyphenylpyruvic acid (Whistance & Threlfall, 1968*a*). Support for this belief comes from the fact that *p*-hydroxyphenylpyruvic acid markedly decreases the incorporation of radioactivity from DL-[β-^{14}C]tyrosine (containing 74% of the radioactivity in the D-isomer) into plastoquinone by maize shoots (Whistance & Threlfall, 1968*a*).

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