On the Biosynthesis of the Quinone Ring of Ubiquinone*

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In *Tetrahymena pyriformis* the quinone ring of ubiquinone is derived from neither phenylalanine nor tryptophan and is therefore synthesized *de novo* from nonaromatic precursors. The organism was grown on a fully synthetic medium containing these two amino acids as the only aromatic nutrients. Both are essential for growth. Ring formation does not take place by condensation of acetate units, nor in any likelihood is the shikimic acid pathway operative. It is therefore probable that the quinone ring of ubiquinone is biosynthesized by a hitherto unknown pathway.

Several investigators have studied the origin of the quinone ring of ubiquinone (UQ).[1] Wiss, Gloor, and Weber (1960) found that phenylalanine was probably not a precursor in rats; however, Bentley *et al.* (1961) and Olson *et al.* (1962) showed a preferential incorporation of phenylalanine-U-C^{14} into the quinone of UQ, also in rats. These results do not completely eliminate the possibility that breakdown products of phenylalanine give rise to the observed activity of the UQ. In these experiments the problem studied was whether the quinone ring of UQ in animals was generally derived from phenylalanine or any other aromatic compound, or whether it was synthesized *de novo* by ring closure of some aliphatic compound. Experiments of this nature are profitably done with microorganisms specifically lacking the ability to synthesize aromatic compounds such as phenylalanine and tryptophan. This eliminates the difficulty of using higher animals, where internal pools obscure the metabolism of administered labeled substances. For the present experiments *Tetrahymena pyriformis* was chosen for three reasons: (1) it produces relatively large amounts of UQ (Crane, 1962; Vakirtzi-Lemonias *et al.*, 1963); (2) it can be grown in a completely synthetic medium in which phenylalanine and tryptophan are the only aromatic compounds present in sufficient quantity to serve as possible precursors of UQ; (3) *T. pyriformis* will not grow without the addition of phenylalanine and tryptophan to the medium.

EXPERIMENTAL

Culture Methods.—*Tetrahymena pyriformis* strain W was grown in the synthetic basal medium A of Dewey *et al.* (1950) from which Tween was omitted and the protogen replaced by DL-thioctic acid at 0.04 γ/ml. Glucose and acetate concentrations in this medium were changed in various experiments as indicated below. After growth for 4 days at room temperature in rotating 10-liter bottles containing 2 liters of medium the cells were harvested by centrifugation. In isotope experiments, the labeled substrates were present throughout the growth period.

Uniformly labeled phenylalanine and tyrosine were obtained from Schwarz BioResearch, tryptophan-(benzene)C^{14} from Nuclear-Chicago Corp., and the rest of the compounds from California Corp. for Biochemical Research. The radiochemical purity of labeled phenylalanine was controlled by chromatography and autoradiography.

Isolation of Ubiquinone.—Cells (equivalent to 2–5 g dry wt) were added to 200 ml of a methanolic solution of KOH (15%) and pyrogallol (5%) and refluxed on a boiling water bath for 30 minutes. Methanol was substituted for the more generally used ethanol to avoid the formation of the ethoxy analogs of UQ (Linn *et al.*, 1959). After rapid cooling the saponification mixture was extracted three times with an equal volume of *n*-heptane each time. The combined extracts were rinsed with water and dried over sodium sulfate. The heptane was evaporated under reduced pressure and the residue was taken up in a small volume of ethanol, leaving much of the "sterol" undissolved. The distinctly yellow material was applied to the starting line of a 20 × 20-cm thin-layer chromatogram of silica gel G. After separation in solvent system 1 (Table I) the yellow zone was removed and the adsorbent was eluted with ethanol. The amount of UQ and the radioactivity were measured, after which the compound was subjected to thin-layer chromatography in solvent system 2.

TABLE I

THIN-LAYER CHROMATOGRAPHY OF UQ_8 ON SILICA GEL G

	Solvent System	R_F	Reference
1.	Isooctane–benzene–ethyl acetate, 25:20:5	0.7	—
2.	Isooctane-benzene-acetone, 25:25:1.5	0.5	Shunk, *et al.*, 1961
3.	Isooctane-butanone, 40:10	0.6	—

Ordinarily the UQ was not further purified, since this procedure yielded a product of at least 95% purity. Thin-layer chromatography for a third time (solvent system 3) did not alter the specific activity and the crystallized UQ_8 had the expected molar extinction (Lester *et al.*, 1959) and a melting point of 35.5° (uncorrected).

Determination of Ubiquinone.—The quinone was measured spectrophotometrically by comparing the absorbance of the oxidized and the reduced (borohydride) compound at 275 mμ in ethanolic solution (Lester *et al.*, 1959).

Assay of Radioactivity.—C^{14} was assessed in a windowless Tracerlab flow counter. The substances were spread at "zero thickness" in standard counting planchets. Counting was carried out for a length of time sufficient to reduce the error to less than 10%.

Ozonolysis of Ubiquinone.—UQ was acetylated with acetic anhydride, pyridine, and zinc dust in an atmosphere of nitrogen, and the diacetate obtained was

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[1] Abbreviations used in this paper are as follows: UQ, ubiquinone; DDMP, 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid.

TABLE II
INCORPORATION OF AMINO ACIDS INTO UBIQUINONE BY *Tetrahymena pyriformis*

Modification of Medium	Tracer	Specific Activity (cpm/μmole) Substrate	UQ	Per Cent
None	L-phenylalanine-U-C^{14}	2,705	578	21.5[c]
No acetate		9,380	4,680	49.9
No acetate		10,780	7,620	70.6
No glucose				
Acetate × 4		2,138	277	13.0[d]
Acetate × 4		3,960	440	11.2
Glucose × 3				
None	DL-phenylalanine-β-C^{14}	3,990	1,135	28.4
None	L-phenylalanine-C^{14}OOH	2,710	25	1.0
None	L-tyrosine-U-C^{14}	10,950[a]	770	7.1[a]
		5,200[b]		14.9[b]
None	DL-tryptophan(benzene)C^{14}	11,900	20	0.17
None		10,680	3	0.03

[a] Calculated for tyrosine alone. [b] Calculated for tyrosine plus phenylalanine. [c] Three further experiments gave values between 18.6 and 26.1%. [d] A second experiment gave a value of 15.2%.

TABLE III
OZONOLYSIS OF UBIQUINONE LABELED FROM PHENYLALANINE-U-C^{14}

UQ	DDMP Expected (a)	(b)	Observed
	(cpm/μmole)		
1357	1357	68	23

[a] Assuming all label of the UQ to be contained in the quinone ring. [b] Assuming all label of the UQ to be contained in the 40 carbons of the side chain, of which 38 are split off by ozonolysis.

purified by thin-layer chromatography on silica gel G with a solvent system of isooctane–benzene–ethyl acetate 25:20:5 (R_F 0.6). The diacetate was ozonolyzed for 1 hour in ethyl acetate containing a small amount of water (Bentley *et al.*, 1961).[2] After the solvent was evaporated the residue was taken up in ether and the ether was extracted five times with a small amount of water. The ether was then evaporated and the residue was taken up in acetone to which a small amount of an aqueous solution of $KMnO_4$ (1 mg/mg UQ) and $MgSO_4$ (0.3 mg UQ) was added. Oxidation was permitted to continue for 1 hour at room temperature. After 0.5 volume of water was added the acetone was evaporated and the slurry was clarified by the addition of solid $NaHSO_3$. The solution was brought to *p*H 3 and extracted with chloroform. The desired 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methyl phenylacetic acid (DDMP) was purified by chromatography twice on thin layers of silica gel G. The solvents were isooctane–benzene–ethyl acetate–acetic acid, 15:15:10:1 for the first and 10:10:30:1 for the second. The R_F values obtained were the same as for an authentic sample of DDMP.[3] The compound was further purified by sublimation at 0.2 mm Hg at 120–150° and after crystallization had a melting point of 122° (uncorrected). The compound was assayed both gravimetrically and spectrometrically using the weak absorption at 268 mμ.

[2] This degradation was carried out at dry ice temperature (Rudney, H., 1962, personal communication).

RESULTS

In Table II are the results obtained when phenylalanine labeled in various positions, tyrosine-U-C^{14}, or tryptophan(benzene)C^{14} was added to the medium and the UQ was isolated. The effect of adding acetate or glucose over and above the amounts present in the basal medium as well as the effects of their omission from the medium on the incorporation of label from phenylalanine into UQ are presented. Although the per cent incorporation is far less than the theoretical value (i.e., the same as the substrate value assuming no degradation and incorporation of the fragments into the side chains), it is appreciable, particularly in the absence of acetate or potential sources of acetate. For this reason the UQ obtained from cells fed phenylalanine-U-C^{14} was subjected to ozonolysis. The results (Table III) indicate that the label in the UQ was confined to the side chain. The activity of acetate in diluting the radioactivity incorporated from phenylalanine also points to the same conclusion.

Tyrosine was less readily used for UQ synthesis and tryptophan gave only negligible incorporation (Table II).

[3] Obtained through the generosity of Dr. O. Isler of Hoffmann-La Roche, Basel.

TABLE IV
INCORPORATION OF VARIOUS COMPOUNDS INTO UBIQUINONE

Modification of Medium	Tracer	Specific Activity (cpm/μmole) Substrate	UQ Observed	UQ Theory	Per Cent
None	Acetate-1,2-C^{14}	210	1110	4950[a]	22.4
None	D-glucose-U-C^{14}	199	320	1560[b]	20.5
None		256	360	2000	18.0
Shikimic acid 25 μg/ml		227	447	1780	25.1

[a] Assuming both ring and side chain to be derived from acetate. [b] Assuming the ring to be formed via shikimic acid and the side chain from acetate, which in turn was formed from glucose.

TABLE V
OZONOLYSIS OF UBIQUINONE LABELED FROM ACETATE-1,2-C^{14}

Experiment	UQ	DDMP Expected (a)	DDMP Expected (b)	DDMP Observed
	(cpm/μmole)			
1	149	29	7.4	7.9
2	526	101	22.4	35.6

[a] Assuming all C equally labeled. [b] Assuming all label in side chain (2 C remaining).

These results show that the quinone ring of UQ cannot be derived from an aromatic substance of the medium, but that it is synthesized from aliphatic compounds by ring closure.

Because of the known biosynthesis of aromatic compounds from acetate (Birch, 1960; Bu'Lock, 1961; Threlfall and Glover, 1962), it was then tested as a precursor of UQ. Table IV shows that it actually labeled the UQ to an appreciable extent. It would be expected that acetate would label the side chain of UQ and also that it would be diluted by endogenous sources of acetate. It was, therefore, necessary to degrade the acetate-derived UQ in order to establish whether or not there was any label in the ring. The data in Table V indicates that acetate is utilized for the construction of the isoprenoid side chain only.

The labeling of UQ by glucose is not diluted by the addition of unlabeled shikimic acid.

DISCUSSION

It has been shown that the animal microorganism *Tetrahymena pyriformis* does not utilize the aromatic rings of phenylalanine, tyrosine, or tryptophan for the synthesis of the quinone ring of UQ, though phenylalanine and tryptophan are absolute growth requirements of the organism (tyrosine can replace a part of the phenylalanine requirement). From this and the known composition of the synthetic medium it is concluded that the quinone ring is built up by ring closure and aromatization of some linear precursor(s).

The two known pathways for aromatic ring closure were considered: the shikimic acid pathway and the condensation of acetate units. The former is generally absent from organisms with an animal-like nutrition (requiring phenylalanine, and the like); furthermore, it has been shown that inactive shikimic acid does not dilute the incorporation of tracer from glucose into UQ. For the acetate pathway the results obtained here were clearly negative: the UQ ring is not assembled from acetate units.

Since the quinone of UQ is neither derived from a preformed aromatic compound nor, in any likelihood, built up by one of the common routes of aromatization, it is presumably synthesized by a hitherto unknown pathway. The composition of the medium would suggest that this pathway starts from one of the essential amino acids.

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