Vol. 2, pp 729–742, Academic Press, New York.
Poston, J. M. (1980) *Biochem. Biophys. Res. Commun. 96*, 838–843.
Sylvester, S. R., & Stevens, C. M. (1979) *Biochemistry 18*, 4529–4531.
Tanaka, K., Aberhart, J. D., Amste, J. I., Jenkins, E. E., & Hsu, C. (1980) *J. Biol. Chem. 255*, 7763–7768.
Tran-Dinh, S., Fermandjian, S., Sala, E., Mermet-Bouvier, R., Cohen, M., & Fromageot, P. (1974) *J. Am. Chem. Soc. 96*, 1484–1493.

Identification of 3-Methoxy-4-hydroxy-5-hexaprenylbenzoic Acid as a New Intermediate in Ubiquinone Biosynthesis by *Saccharomyces cerevisiae*†

Robert R. Goewert,‡ C. Jeffrey Sippel,§ Michael F. Grimm,§ and Robert E. Olson*

ABSTRACT: A ubiquinone-deficient mutant strain of *Saccharomyces cerevisiae*, 26H, was found to accumulate a previously unidentified intermediate in ubiquinone biosynthesis when grown in the presence of *p*-hydroxy[7-^{14}C]- or -[U-^{14}C]benzoic acid. This intermediate was isolated from the lipid extracts of a 100-L culture of 26H and purified by various chromatographic procedures to yield 20 mg of product. Analysis by means of NMR, IR, UV, and mass spectrometry revealed the structure of this new intermediate to be 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid (3-MHHB). In vitro experiments with isolated yeast and rat mitochondria showed that 3-MHHB could be converted to ubiquinone-6. These findings indicate that 3-O-methylation precedes decarboxylation of the prenylated protocatechuic acid intermediate in the biosynthesis of ubiquinone in eukaryotes.

The pathway for the biosynthesis of the electron-transport component ubiquinone has been described for prokaryotes by using mutants of *Escherichia coli* (Gibson & Young, 1978). For eukaryotes, however, the pathway from 4-hydroxy-5-polyprenylbenzoate to 6-methoxy-2-polyprenylphenol is thought to be different from that of prokaryotes. The compound 3,4-dihydroxy-5-hexaprenylbenzoic acid has been identified as an intermediate in the mitochondrial biosynthesis of ubiquinone by the lower eukaryote, *Saccharomyces cerevisiae* (Goewert et al., 1978). This intermediate was found to accumulate in methionine auxotrophs deprived of methionine, suggesting that methylation of the protocatechuate intermediate via *S*-adenosylmethionine, was the next step in the reaction sequence (Goewert et al., 1981). Additional support for this pathway comes from the work of Nambudiri et al. (1977), who found that mitochondrial preparations from rat heart and liver were able to prenylate both 3,4-dihydroxybenzoic acid and 3-methoxy-4-hydroxybenzoic acid. Other investigators (Casey & Threlfall, 1978) have shown that yeast mitochondria, when incubated with the ubiquinone precursors 4-hydroxybenzoate and isopentenypyrophosphate, or 3-hexaprenyl-4-hydroxybenzoate, synthesize 6-methoxy-2-hexaprenylphenol, 5-demethoxyubiquinone-6, and ubiquinone-6. Unfortunately the more labile carboxylated intermediates were not detected under their experimental conditions.

In this paper we report the identification of 3-methoxy-4-hydroxy-5-hexaprenylbenzoate, a new intermediate in the biosynthesis of ubiquinone-6. This compound was found to accumulate in a strain of *Saccharomyces cerevisiae* unable to synthesize ubiquinone-6. A preliminary report of this work has been made (Goewert et al., 1978). In addition, we have found that both yeast and rat mitochondria readily convert this compound to ubiquinone, demonstrating that the compound is the main pathway for ubiquinone biosynthesis in both higher and lower eukaryotes.

Materials and Methods

Male albino rats of either the Sprague-Dawley or Wistar strains, weighing 150 g and fed a stock commercial diet (Purina Laboratory Chow, Ralston Purina Co., St. Louis, MO) ad libitum, were used in these studies.

All chemicals were at least reagent grade: aluminum oxide from Aleysharm Chemicals and both analytical (0.25 mM) and preparative (2 mm) silica gel-60 thin-layer chromatography plates from Brinkmann, and cytochrome *c*, glutathione (reduced), dithiothreitol, 3-methoxy-4-hydroxybenzoic acid, and vanillic acid from Sigma. Ubiquinone standards and solanesol were the generous gifts of Dr. O. Wiss of Hoffmann-La Roche Ltd. (Basel, Switzerland). All ubiquinone pathways intermediates were synthesized following the procedure described by Nowicki et al. (1972). [*methyl*-^{3}H]-Methionine was purchased from New England Nuclear. *p*-Hydroxy[G-^{3}H]benzoic acid (800 mCi/mmol) was obtained from Amersham-Searle by custom synthesis under the direction of Dr. Bryan W. Baker. This product was purified to constant specific radioactivity by thin-layer chromatography (Nowicki et al., 1972). *p*-Hydroxy[U-^{14}C]benzoate was synthesized by sodium fusion of [U-^{14}C]tyrosine according to the method of Ho et al. (1973). *p*-Hydroxy[7-^{14}C]benzoate was prepared by catalytic reduction of *p*-benzyloxy[*carbox*-

† From the Edward A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104. *Received February 2, 1981.* This investigation was supported in part by Grant AM 10004 from the National Institutes of Health, U.S. Public Health Service.

‡ Present address: Departments of Medicine and Pathology, Washington University, St. Louis, MO 63110. Parts of this paper were taken from a thesis submitted by R.G.G. to St. Louis University in partial fulfillment of the requirements for the Ph.D. degree.

§ Present address: Students of Medicine at St. Louis University, St. Louis, MO 63104.

0006-2960/81/0420-5611$01.25/0

yl-^{14}C]benzoic acid (Amersham) and purified to >99.9% by TLC.[1]

Nuclear magnetic resonance (NMR) spectra were obtained by using a Varian HA 60 MC NMR spectrometer, with deuterated chloroform (Aldrich Chemical Co.) as the solvent and tetramethylsilane as an internal standard. Infrared absorption spectra (IR) were obtained by using a Model 24 Perkin-Elmer double beam spectrophotometer. Mass spectra were obtained by using a modified medium-resolution LKB Model 9000 single-focusing computer-interfaced mass spectrometer. The direct probe was used to introduce samples at a vacuum of 10^{-8} torr, ionizing energy of 70 eV, ionizing current of 240 μA, and accelerating energy of 3.5 kV. The temperature of the direct probe was varied from ambient to 145 °C.

Growth and Labeling of Yeast. Yeast strain 26H was grown in defined minimal media at 31 °C on a rotary shaker (Goewert et al., 1981). Growth was monitored by using a Klett–Summerson nephelometer with a No. 42 filter. During early exponential growth (20–30 Klett units), cultures were inoculated with the ubiquinone precursors [U-^{14}C]pHBA or [G-^{3}H]pHBA. After a density of 200 Klett units (early log phase) was attained, the cells were collected by centrifugation, washed, and then used in the experiments described.

Preparation of Yeast and Rat Liver Mitochondria. Yeast mitochondria were isolated from 2 L of the prototrophic strain D273-10B according to the method of Lang et al. (1977). Liver mitochondria were prepared from rats weighing 120–150 g which were fasted overnight, decapitated, and exsanguinated. The liver was quickly removed and placed in 80 mL of cold wash medium composed of 250 mM sucrose, 5 mM Tris-HCl, and 1 mM Tris–EGTA adjusted to pH 7.2 at 4 °C (all subsequent steps were performed at 4 °C). The liver was minced and the supernatant decanted, followed by repeating the washing with an additional 80 mL. Homogenization was performed in a 50-mL glass homogenizer with a Teflon pestle (A. H. Thomas Co.) connected to a drill press by four 15-s strokes at 1000 rpm. The homogenates were centrifuged for 5 min at 2500 rpm in a Sorvall RC2-B centrifuge using a SS-34 rotor. The pellets were discarded and the supernatant, including one-half of the light brown fluffy layer, was discarded. The remaining pellet was resuspended in a buffer composed of 250 mM sucrose and 5 mM Tris-HCl at pH 7.2. Centrifugation was performed as before. The supernatant was discarded, and the light layer was decanted twice, leaving a dark brown pellet. The final pellet was resuspended to a concentration of 35–40 mg of protein per mL, as determined by the method of Lowry et al. (1951).

Extraction Procedure. Harvested cells were washed once with distilled H_2O by centrifugation at 500*g* for 10 min at 4 °C. The pellet was lyophilized overnight, weighed, and then finely ground with a mortar and pestle. The pulverized cells were extracted with 100 mL of acetone per g dry weight of cells for 12 h at 4 °C. An equal volume of peroxide-free anhydrous diethyl ether was added, and the cells were extracted as noted above for another 12 h. The extraction liquor was separated from the cellular debris by filtration and the above schedule repeated. The two extracts were pooled and reduced in volume to several milliliters by rotary evaporation at 40 °C. The remaining extract was concentrated to a viscous lipid under a gentle stream of N_2 and analyzed for ubiquinone or intermediates by TLC. The recovery of ubiquinone-related compounds by this procedure was found in close agreement with the method of Gordon & Stewart (1969) as determined by both spectrophotometric and radioactive quantitation of ubiquinone-6 isolated from [U-^{14}C]pHBA labeled cultures of D273-10B.

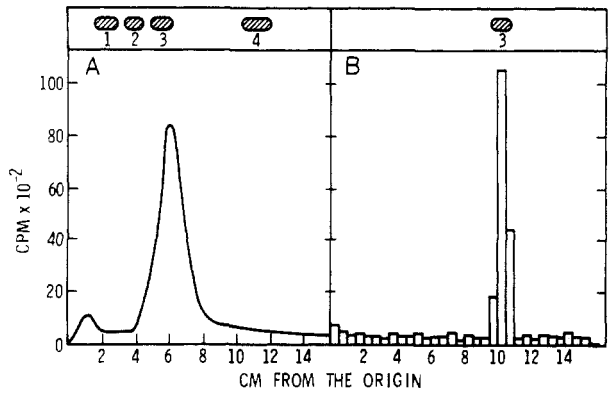

FIGURE 1: Radiochromatographic profile of crude and purified ^{14}C-labeled lipid band from 26H. (A) Radiochromatography of TLC plate (silica gel 0.25 mm) of acetone/ether extract of yeast mutant 26H grown in the presence of *p*-hydroxy[U-^{14}C]benzoate developed with cyclohexane/ether/acetic acid (50:50:0.5). A strip-counter was used. Markers are (1) pHBA, (2) 3,4-dihydoxy-5-nonaprenylbenzoate, (3) 3-methoxy-4-hydroxy-5-nonaprenylbenzoate, (4) 2-nonaprenylphenol. (B) Radiochromatogram of TLC plate of purified 3-methoxy-4-hydroxy-5-hexaprenylbenzoate developed with benzene/acetone (1:1). Increments of 0.5 cm were scraped and counted in a scintillation spectrometer.

Detection of Phenolic Intermediates on TLC Plates. Phenolic intermediates and synthetic products were detected with Gibb's reagent (Whistance & Threlfall, 1967) eliciting a characteristic blue spot. Compounds having aromatic substitutions at both the para and meta positions demonstrate a characteristic light brown color.

Mitochondrial Incubations. Mitochondria were incubated for 3 h at 37 °C in 50-mL flasks containing 5 mL of a mixture of 0.33 M mannitol, 0.66 M sorbitol, 13 mM Tris, 0.0006 M EDTA (potassium), 9.7 mM glutathione (reduced form), 3.4 mM malic acid, 3.3 mM ATP (pH 8), 5.1 mM *S*-adenosylmethionine, 0.5 mg/mL cytochrome *c* (horse heart), and 2 mg/mL bovine serum albumin at pH 7.4 which was freshly prepared and filter sterilized.

Results

Detection of a Novel Benzoate-Type Intermediate Less Polar Than 4-Hexaprenyl-4-hydroxybenzoic Acid. The detection of intermediates in ubiquinone biosynthesis in the ubiquinone-deficient strain 26H (De Kok et al., 1975) was investigated by growing two cultures of 26H, one in the presence of the ubiquinone precursor 4-hydroxy[U-^{14}C]benzoic acid (pHBA) and the other in the presence of [7-^{14}C]pHBA. Analysis of the lipid extracts from these late exponential cultures revealed the accumulation of a radiolabeled compound characterized by a unique chromatographic behavior. Further TLC analysis demonstrated this compound to be significantly more polar than ubiquinone-6 but less polar than any known benzoate-type ubiquinone intermediate (Figure 1). The novel metabolite gave a positive blue color reaction with Gibb's reagent, indicating carboxyl substitution para to a phenolic group (Whistance et al., 1967).

When wild-type D273-10B and the ubiquinone-deficient strain 26H were grown in a defined media including [*methyl*-^{3}H]methionine for two generations, a 4-fold greater incorporation into ubiquinone in D273-10B was found as com-

[1] Abbreviations used: *p*-HBA, 4-hydroxybenzoic acid; TLC, thin layer chromatography; Q_6, ubiquinone-6; 3,4-DHHB, 3,4-dihydroxy-5-hexaprenylbenzoic acid; 3-MHHB, 3-methoxy-4-hydroxy-5-hexaprenylbenzoate.

Table I: Large-Scale Isolation of 3-Methoxy-4-hydroxy-5-hexaprenylbenzoic Acid from *S. cerevisiae* Strain 26H[a]

step	lipid (mg)	radioactivity (dpm × 10^{-6})	sp act. (dpm per mg × 10^{-4})
extract	5320	7.64	0.14
hexane soluble	1390	6.17	0.44
alumina column, 4–6% ether	73.1	4.62	6.32
preparative TLC, benzene/acetone (1:1)	19.3	4.15	21.5
preparative TLC, ether/cyclohexane (8:2)	18.2	4.07	22.3

[a] 26H (100 L) was grown 4–6 h into stationary phase in SD media containing 25 μCi of [7-^{14}C]pHBA. The cells were harvested, lyophilized, extracted, and chromatographed as described under Materials and Methods.

pared to evidence of the metabolite in strain 26H. These results indicate a methyl group ratio of 4:1 for ubiquinone/26H metabolite. It should be noted that Nowicki et al. (1972) also demonstrated the incorporation of the methyl group from [*methyl*-^{14}C]methionine into 6-methoxy-2-nonaprenylphenol in rat liver slices. Furthermore, by growing 26H in the presence of both [7-^{14}C]pHBA and [*methyl*-^{3}H]methionine, a doubly labeled metabolite was produced. From the known specific activities of the precursor, it was calculated that the intermediate contained one methyl and one carboxyl group per molecule.

Isolation and Identification of 3-Methoxy-4-hydroxy-5-hexaprenylbenzoic Acid (3-MHHB). One hundred liters of 26H was grown in batch culture in SD media at 31 °C to a density of 280 Klett units, and 25 μCi of [7-^{14}C]pHBA was inoculated into one culture flask to serve as a radioactive marker throughout the isolation schedule. The cells were harvested and extracted as described under Materials and Methods; 5.32 g of acetone/ether crude lipid extract containing 7.64×10^6 dpm of ^{14}C label was obtained from the lyophilized cells. This crude lipid fraction was dissolved in 300 mL of hexane and then concentrated to 60 mL by rotary evaporation. An off-white amorphous precipitate formed during this reduction in volume. The hexane fraction was separated from the precipitate and reduced to an oily residue by rotary evaporation at room temperature. This volume-reduction step made use of the differential solubility of the various ether/acetone-soluble compounds in hexane, thereby eliminating greater than 75% of the starting materials, yet retaining 80% of the extracted label. The hexane portion was next taken to dryness, and the residue was redissolved in petroleum ether and chromatographed on an alumina column by stepwise elution with increasing amounts of diethyl ether in petroleum ether, similar to a procedure described by Gold & Olson (1966). The data for this and subsequent steps are summarized in Table I. A large peak of radioactivity containing 4.62×10^6 dpm was eluted in the 4–6% ether fraction, with a smaller peak eluting with 2% ether.

The two peaks were separately pooled, reduced in volume, and analyzed by TLC with solvent system 1 (Table II). Radioactivity from the 2% ether fraction demonstrated an R_f of 0.20, which corresponds in position to 3,4-dihydroxyhexaprenylbenzoate (3,4-DHHB), a recently identified ubiquinone intermediate (Goewert et al., 1977, 1981). The radioactivity from the 4–5% ether fractions comigrated with the new metabolite at R_f 0.33, and in addition, this compound was observed to develop the characteristic blue reaction color produced using Gibb's reagent.

The material from the 4–5% ether fraction was next chromatographed by using preparative silica gel G TLC plates developed in solvent system 2; 19.3 mg of material was recovered, containing 4.15×10^6 dpm from a single band migrating to R_f 0.65. This highly purified 3-MHHB was finally rechromatographed in solvent system 1 and yielded 18.2 mg of purified compound which was found to have mp 31–32 °C. It was greater than 95% homogeneous, as demonstrated by multiple TLC in the various solvent systems. This highly purified compound was next used for chemical and spectral analysis.

Table II: R_f[a] of Ubiquinone and Ubiquinone Biosynthetic Intermediates in Various TLC Systems

compound	I	II	III	IV	V
4-hydroxybenzoic acid	0.40	0.10	0.00	0.00	0.18
3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid	0.65	0.75	0.33	0.46	0.90
3,4-dihydroxy-5-hexaprenylbenzoic acid	0.57	0.65	0.20	0.14	0.72
3,4-dihydroxy-5-nonaprenylbenzoic acid	0.57	0.65	0.20	0.14	0.72
4-hydroxy-5-nonaprenylbenzoic acid	0.73	0.70	0.27	0.21	0.81
ubiquinone-6	0.82	0.92	0.61	0.53	S.F.
ubiquinone-9	0.82	0.92	0.61	0.53	S.F.

I benzene/acetone (1:1)
II benzene/chloroform (1:1)
III cyclohexane/diethyl ether/acetic acid (50:50:0.5)
IV cyclohexane/diethyl ether (8:2)
V benzene/acetone/ammonium hydroxide (6:2:2)

[a] Silica gel G (Brinkman) analytical TLC plates (5 × 20 cm; 250 μm) were developed in 15 cm in the above solvent systems.

Mass Spectra. The mass spectra for this compound showed a molecular ion at *m/e* 576 and its decarboxylated product ($M^+ - 44$) at 532. Two stable benzylic ring fragments were seen at *m/e* 235 and 181 and side-chain hydrocarbon fragments for $C_5H_9^+$ and $C_6H_9^+$ at *m/e* 68 and 81, respectively. Treatment of this new metabolite with diazomethane generated a single radioactive product, as ascertained by TLC. The mass spectra of this product demonstrated a molecular ion at *m/e* 590 which readily loses methoxy and ethyl ester fragments at *m/e* 559 and 531, respectively. The comparative mass spectra for the natural product, its methyl ester, and the corresponding nonaprenyl homologue are shown in Figure 2.

NMR and IR Spectra. Key structural features of the natural product are revealed by its NMR spectrum shown in Figure 3. A singlet at δ 3.88 corresponds to the resonance contributed by the 3 methoxy protons. The other peaks at δ 6.85, 4.17, and 2.0–1.61 are attributed to aromatic, vinylic, and alkyl protons, respectively. The infrared absorption spectrum for 3-MHHB is shown in Figure 4. The spectrum displays absorption bands characteristic for both its isoprenyl side chain and its aromatic nucleus. Infrared spectra data taken together support a structure for this new metabolite of 3-methoxy-4-hydroxy-5-hexaprenylbenzoate.

Conversion of 3-MHHB into Ubiquinone-6 in the Isolated Mitochondria from S. cerevisiae and the Rat. To establish a precursor–product relationship of 3-MHHB to ubiquinone-6 in yeast, the in vitro conversion of [*ring*-^{14}C]3-MHHB to [*ring*-^{14}C]ubiquinone-6 was studied with isolated mitochondria from wild-type strain D273-10B.

[*ring*-^{14}C]3-MHHB was purified from [U-^{14}C]pHBA labeled cultures of 26H as previously described and purified to a constant specific activity of 1.86 μCi/mmol; 30 μg of [*ring*-^{14}C]3-MHHB ($2.15^4 \times 10^5$ cpm) was added to a flask containing D273-10B mitochondria (27.5 mg of protein), incubated for 3 h, lyophilized, extracted, and analyzed for ra-

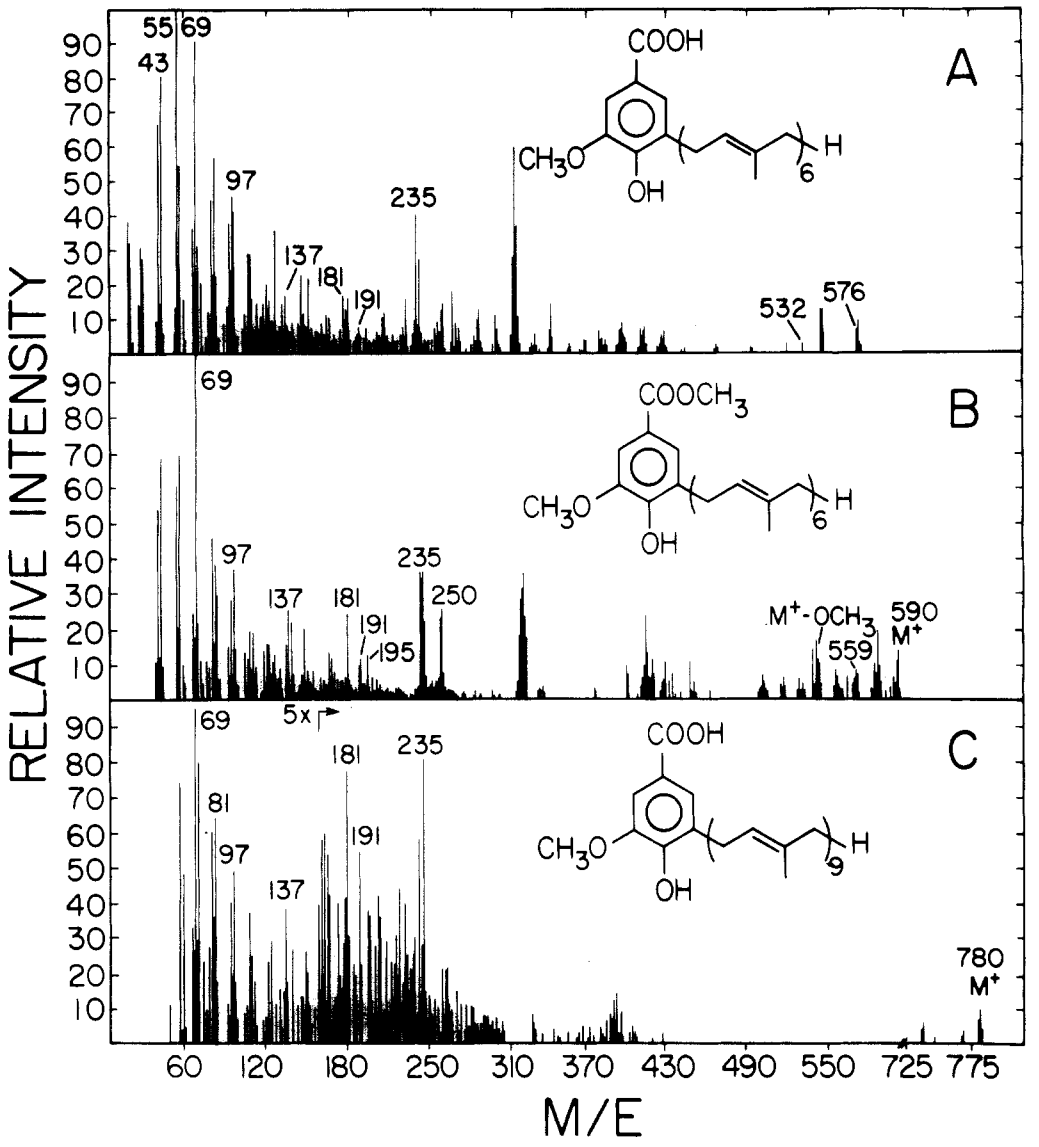

FIGURE 2: Medium-resolution mass spectra of homologues and analogues on prenylated vanillic acid derivatives obtained by direct inlet technique: (A) 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid; (B) 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid methyl ester; (C) 3-methoxy-4-hydroxy-5-nonaprenylbenzoic acid.

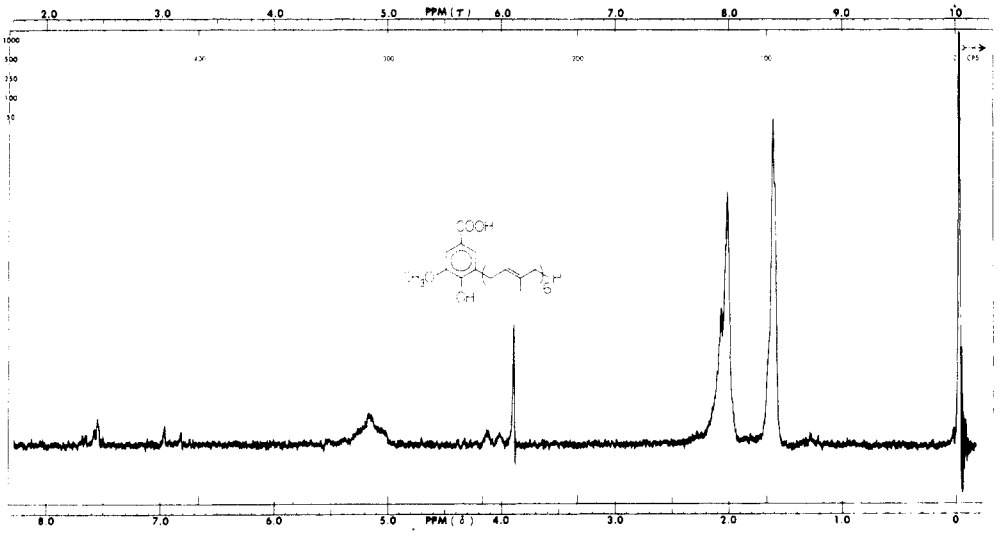

FIGURE 3: Nuclear magnetic resonance spectrum of 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid. Natural product (18.2 mg) was dissolved in 0.2 mL of $CDCl_3$ (99.8 mol % deuterium) and placed in a NMR microspin tube. The spectrum obtained at ambient temperature was a frequent response of 20 Hz, sweeptime of 250 s, sweep width of 500 Hz, sweep offset of 500 Hz, oscillator frequency of 0.02 MG, and sweep frequence of 0.50 MG. Trimethylsilane was included as an internal standard.

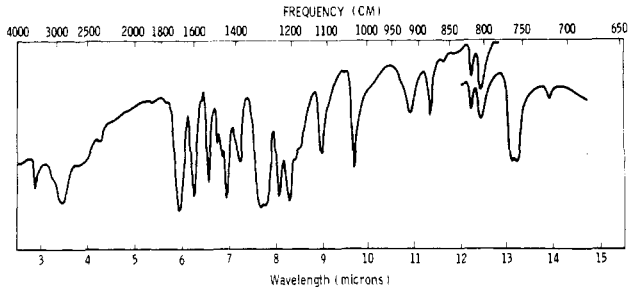

FIGURE 4: Infrared absorption spectrum of 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid. 3-MHHB (1 mg) was ground in a mortar with 50 mg of anhydrous KBr (IR grade). A KBr disk was prepared and spectra were immediately obtained. The absorption bands were at 3480, 2920, 1690, 1610, 1530, 1470, 1390, 1290, 1210, 820, and 760 cm^{-1}.

Table III: In Vitro Conversion of 3-MHHB to Ubiquinone-6 in Isolated Mitochondria from *S. cerevisiae* Strain D273-10B and Rat Liver[a]

	3-MHHB (dpm × 10^{-5})		ubiquinone-6 (dpm × 10^{-4})	
step	yeast	rat	yeast	rat
incubation	2.15	1.00		
crude acetone/ether fraction chromatographed (TLC) in cyclohexane/ether/HOAc (50:50:0.05)	1.48	0.78	4.82	1.62
TLC ubiquinone fraction in benzene/chloroform (1:1)			2.85	1.00
TLC ubiquinone fraction in benzene/acetone (1:1)			1.85	0.84

[a] [*ring*-^{14}C]3-MHHB was isolated from [U-^{14}C]pHBA-labeled *S. cerevisiae* 26H. [*ring*-^{14}C]3-MHHB was purified to a constant specific activity of 1.86 μCi/mol as described under Materials and Methods. Yeast mitochondria were isolated from 2 L of the strain D273-10B by a modification of the method of Lang et al. (1977) and from rat liver as described under Materials and Methods. Two 5-mL incubation mixtures in 50-mL flasks containing 0.33 M mannitol, 0.66 M sorbitol, 0.13 M Tris, 0.0006 M EDTA (potassium), 9.7 mM glutathione (reduced form), 3.4 mM malic acid, 3.26 mM ATP (pH 8), 5.06 mM *S*-adenosylmethionine, 0.5 mg/mL cytochrome *c* (horse heart), and 2 mg/mL bovine serum albumin at pH 7.4 were freshly prepared and filtered sterilized. Mitochondria (27.5 mg of mitochondrial protein) were added to both flasks; 30 μg of [*ring*-^{14}C]3-MHHB (2.154 × 10^5 dpm) was added to both flasks and the mixture allowed to incubate at 30 °C for 3 h. The reaction mixtures were next lyophilized and acetone/ether extracted. The crude lipid extracts were analyzed by TLC.

dioactive products by TLC. A new radioactive peak was found to comigrate with the endogenous ubiquinone-6 (visual identification of yellow band). Approximately 60% of the 3-MHHB was recovered from each sample, with nearly 20% of the initial counts in both samples comigrating with ubiquinone-6. The ubiquinone-6 band was isolated from the TLC plate and sequentially chromatographed in solvent systems 2 and 4. The major portion of radioactivity applied was found to comigrate with ubiquinone-6 in both solvent systems. A summary for this in vitro conversion experiment is presented in Table III.

In most preparations, 1 mg of mitochondrial protein converted 0.39 nmol of 3-MHHB to ubiquinone-6 during the 3-h incubation period. Deletion of either SAM or glutathionine (reduced form) from the incubation mixture resulted in less than 10% conversion of 3-MHHB to ubiquinone-6.

Rat liver mitochondrial preparations, as described under Materials and Methods, were similarly tested for its ability to convert 3-MHHB to ubiquinone-6; 15 μg (100 000 cpm) of [*ring*-^{14}C]3-MHHB was incubated for 3 h with yeast and rat liver mitochondria. Following a purification scheme similar

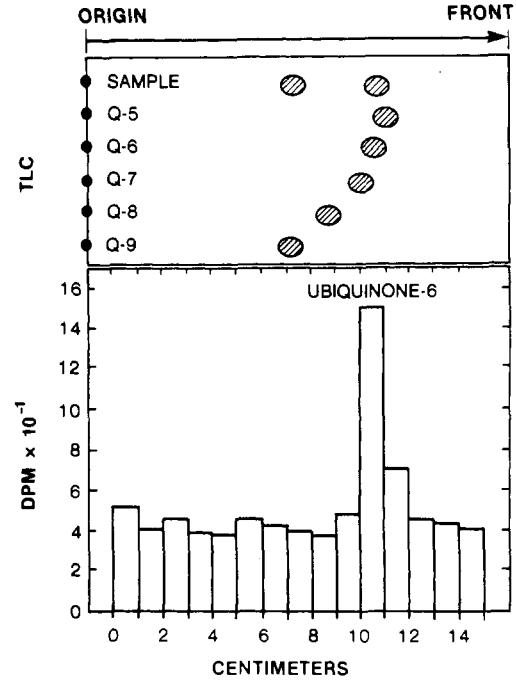

FIGURE 5: Reverse-phase TLC of ubiquinone 4-10 with radioactive ubiquinone from "in vitro" conversion of 3-MHHB by rat liver mitochondria. A 5% paraffin-impregnated silica gel G TLC plate was developed with acetone/H_2O (95:5). The sample slot containing the radioactive ubiquinone sample was analyzed by scraping and counting every 1 cm from origin to the solvent front in Biofluor.

to that outlined for yeast, rat liver mitochondria was found to convert approximately 10% of the exogenously added [*ring*-^{14}C]3-MHHB to ubiquinone-6. In the same experiment, yeast was found to convert approximately 15% of the [*ring*-^{14}C]3-MHHB to ubiquinone-6.

Proof of the structure of the radioactive ubiquinone isolated from rat mitochondria was accomplished by reverse-phase TLC using 5% paraffin-impregnated silica gel plates, developed with acetone/H_2O (95:5). Excellent separation of ubiquinones-4 through -10 was achieved by this method. Radioactivity was found to comigrate with ubiquinone-6 and not with the nonaprenyl homologue which is endogenous in the rat, as shown in Figure 5. Unequivocal proof of the radioactive product's identity was provided by mass spectrometry, where the radioactive sample spectra was found to be identical with that of ubiquinone-6 isolated from yeast.

Discussion

Previous research in our laboratory has demonstrated that methionine biosynthetic auxotrophs accumulate 3,4-dihydroxy-5-hexaprenylbenzoate when starved for methionine in the presence of [U-^{14}C]- or [7-^{14}C]pHBA (Goewert et al., 1981). Addition of methionine to the culture reactivated ubiquinone synthesis and stimulated normal growth. The in vivo observations indicated that the blocked step was a methylation. Since 6-methoxy-2-hexaprenylphenol is an established intermediate in ubiquinone biosynthesis (Casey & Threlfall, 1978; Nowicki et al., 1972), a logical intermediate between 3,4-DHHB and 6-methoxy-2-hexaprenylphenol would be 3-methoxy-4-hydroxy-5-hexaprenylbenzoate.

Ubiquinone-deficient yeast strain 26H was observed to accumulate a previously unidentified compound when grown in the presence of the labeled ubiquinone precursors [U-^{14}C]- or [7-^{14}C]pHBA. In addition, this benzoate-type intermediate was found to be labeled by [*methyl*-^{3}H]methionine, presumably derived via *S*-adenosylmethionine. This relatively stable

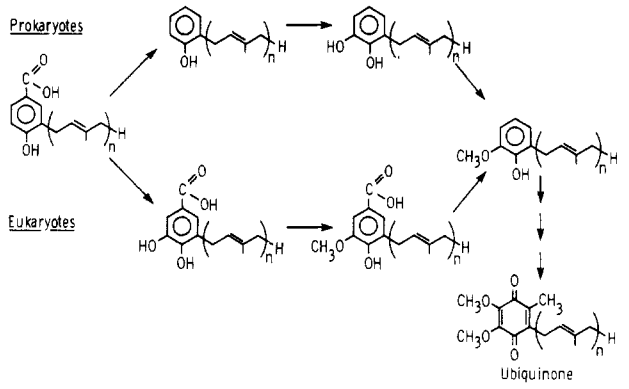

FIGURE 6: Divergent and common pathways for ubiquinone biosynthesis in eukaryotic and prokaryotic organisms.

methylated intermediate was accumulated in sufficient quantities in strain 26H to permit 20 mg of the natural product to be isolated and identified.

The mass spectrum of this natural product demonstrated large peaks at *m/e* 69, 81, and 95 characteristic for isoprenoid-type compound (Muraca et al., 1967). Peaks at *m/e* 97, 181, and 235 correspond to the decarboxylated vanillate, tropylium, and chromenylium ions, respectively. A molecular ion was identified at *m/e* 576, which agreed with the calculated molecular weight for 3-methoxy-4-hydroxy-5-hexaprenylbenzoate. A mass spectrum of the synthetic nonaprenyl homologue demonstrated a nuclear fragmentation pattern identical with that of the natural product. The NMR spectrum was furthermore consistent with the proposed structure. Additional evidence from chromatographic properties and IR spectral studies verified the structure of this natural product as 3-methoxy-4-hydroxy-5-hexaprenylbenzoate.

In vitro experiments with isolated yeast and rat mitochondria demonstrated the conversion of 3-methoxy-4-hydroxy-5-hexaprenylbenzoate to ubiquinone-6. The conversion was found to be SAM dependent, requiring a sulfhydryl reducing agent for good conversion. The fact that rat mitochondria synthesized ubiquinone-6 from 3-MHHB indicated that all of the enzymes required to convert 3-MHHB to ubiquinone-6 are relatively nonspecific with respect to the isoprenyl side chain.

It is now clear that the pathway for ubiquinone biosynthesis in prokaryotic organisms differs from that in eukaryotic organisms (Cox et al., 1969; Raman et al., 1969; Nowicki et al., 1972). As shown in Figure 6, prokaryotic organisms convert 4-hydroxy-5-polyprenylbenzoate to 6-methoxy-2-polyprenylphenol, a sequence of steps consisting of decarboxylation, hydroxylation, and methylation. Eukaryotic organisms on the band metabolize pHBA in a sequence involving hydroxylation, methylation, and finally decarboxylation.

The methylation of 3,4-dihydroxy-5-hexaprenylbenzoate to 3-methoxy-4-hydroxy-5-hexaprenylbenzoate is regulated in yeast mitochondria. Recent experiments in our laboratory using wild-type strains have shown that the low steady-state levels of 3,4-DHHB rose to high levels when glucose in the medium was increased from 1 to 5%. This change in the level of 3,4-DHHB was accompanied by a decrease in rate of ubiquinone biosynthesis (Sippel et al., 1979). Studies of the mechanism of this regulation are in progress.

Acknowledgments

We thank Dr. Ronald Cockrell for helpful advice concerning isolation of rat liver mitochondria and acknowledge the expert technical assistance of William Frasure in operating the LKB 9000 mass spectrometer.

References

Casey, J., & Threlfall, D. R. (1978) *FEBS Lett. 85*, 249.
Cox, B. G., Young, I. G., McCann, C. M., & Gibson, R. (1969) *J. Bacteriol. 99*, 450.
De Kok, J., & Slater, E. C. (1975) *Biochim. Biophys. Acta 376*, 27.
Gibson, F., & Young, I. G. (1978) *Methods Enzymol. 53*, 600.
Goewert, R. R., Sippel, C. J., & Olson, R. E. (1977) *Biochem. Biophys. Res. Commun. 77*, 599.
Goewert, R. R., Sippel, C. J., Grimm, M. F., & Olson, R. E. (1978) *FEBS Lett. 87*, 219.
Goewert, R. R., Sippel, C. J., & Olson, R. E. (1981) *Biochemistry 20*, 4217.
Gold, P., & Olson, R. E. (1966) *J. Biol. Chem. 241*, 3507.
Gordon, P. A., & Stewart, P. R. (1969) *Biochim. Biophys. Acta 177*, 358.
Ho, J., Nilsson, J. L. Y., Skelton, F. S., & Folkers, K. I. (1973) *J. Org. Chem. 38*, 1059.
Lang, B., Burger, G., Doxiadis, I., Thomas, D. Y., Banllow, W., & Kauchewitz, K. F. (1977) *Anal. Biochem. 77*, 110.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Muraca, F. R., Daves, G. D., & Folkers, K. (1967) *J. Am. Chem. Soc. 89*, 1505.
Nambudiri, A. M. D., Brockman, D., Alan, S. S., & Rudney, H. (1977) *Biochem. Biophys. Res. Commun. 76*, 282.
Nowicki, H. G., Dialameh, G. H., & Olson, R. E. (1972) *Biochemistry 11*, 896.
Raman, T. S., Rudney, H., & Buzzelli, W. K. (1969) *Arch. Biochem. Biophys. 130*, 164.
Sippel, C. J., Goewert, R. R., & Slachman, F. N. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 508.
Whistance, G. R., & Threlfall, D. R. (1967) *Biochem. Biophys. Res. Commun. 28*, 295.