Identification of 3,4-Dihydroxy-5-hexaprenylbenzoic Acid as an Intermediate in the Biosynthesis of Ubiquinone-6 by *Saccharomyces cerevisiae*†

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ABSTRACT: The mutant strain of *Saccharomyces cerevisiae* E3-24 is unable to synthesize ubiquinone-6. When this mutant is grown in the presence of *p*-hydroxy[U-^{14}C]benzoate or *p*-hydroxy[*carboxy*-^{14}C]benzoate, a radioactive compound accumulates. This new metabolite has been isolated and identified as 3,4-dihydroxy-5-hexaprenylbenzoate (3,4-DHHB). Aerobically grown prototrophic strains of *S. cerevisiae* were found to contain only low levels of this compound. When strain X963-18C, blocked at homoserine *O*-transacetylase (in methionine biosynthesis), was deprived of methionine, ubiquinone biosynthesis ceased, and 3,4-DHHB was observed to accumulate. This suggested that *S*-adenosylmethionine (SAM) could be the methyl donor for 3,4-DHHB. Restoration of methionine to the cultures released this block and resulted in the conversion of 3,4-DHHB to ubiquinone-6, demonstrating a precursor–product relationship. The identification of 3,4-DHHB as an intermediate in ubiquinone biosynthesis in yeast establishes an alternate pathway for ubiquinone biosynthesis in eukaryotes.

The use of mutants for characterizing metabolic pathways is a powerful and widely used tool. Gibson and collaborators (Gibson, 1973) have elucidated the biosynthetic pathway for ubiquinone-8 in *Escherichia coli* by selecting ubiquinone-deficient mutants and identifying the intermediates which accumulated at various blocked reaction steps in the pathway. The pathway described for *Escherichia coli* is identical with the pathway in *Rhodospirillum rubrum* proposed earlier (Fris et al., 1966) which is based on the logical ordering of a series of prenylated phenol and quinones, identified by the mass spectrometry of bacterial lipid extracts. Other investigators have examined various microorganisms for the detection of phylogenetic differences in the ubiquinone biosynthetic pathway. Most investigators reported the isolation of previously identified intermediates, indicating that for prokaryotes, in general, ubiquinone is biosynthesized by the same pathway (Whistance et al., 1969, 1970; Imamota & Sehoh, 1967).

The biosynthetic pathway for ubiquinone in eukaryotes is not as well characterized. In this laboratory, 5-methoxy-2-nonaprenylphenol (Nowicki et al., 1972), 5-demethoxyubiquinone-9 (Trumpower et al., 1972), and 5-demethylubiquinone-9 (Houser & Olson, 1974) have been shown to be bonafide intermediates in ubiquinone biosynthesis in the rat. On the other hand, nonaprenylphenol, an intermediate in the biosynthesis of ubiquinone-9 by prokaryotes, was found not to be an intermediate in the rat (Trumpower et al., 1974). Thus, it appeared that although prokaryotes and eukaryotes share portions of this biosynthetic pathway, significant differences exist between them. In both pro- and eukaryotes, *p*-hydroxybenzoate (*p*HBA)[1] has been demonstrated to be the first metabolite in the pathway (Rudney & Parson, 1963; Olson et al., 1963). The metabolic origin of *p*HBA, however, is known to be different for pro- and eukaryotes. Prototrophic prokaryotes convert chorismic acid to *p*HBA and pyruvate, whereas in certain higher plants, *p*HBA is derived from *p*-coumaric acid. Higher eukaryotes, which are unable to synthesize aromatic amino acids, obtain *p*HBA from tyrosine (Olson, 1966). Eukaryotes have further specialized this pathway by compartmentalization of the site for ubiquinone biosynthesis to the mitochondria, where ubiquinone also functions as a central component in electron transport (Trumpower et al., 1974). A separate pathway for *p*HBA metabolism has been proposed for the rat (Trumpower et al., 1974) in which hydroxylation to form poly(prenyl)protocatechuate may precede decarboxylation. Support for this pathway comes from recent evidence in which protocatechuate was found to be converted to ubiquinone in rat heart and liver mitochondria (Nambudiri et al., 1977).

We report here evidence for this alternate pathway in eukaryotic organisms by the isolation and identification of 3,4-dihydroxy-5-hexaprenylbenzoic acid as an obligate intermediate in the ubiquinone biosynthetic pathway of *Saccharomyces cerevisiae*. A preliminary report of this study has been made (Goewert et al., 1977).

Materials and Methods

Reagents. Yeast nitrogen base without amino acids and ammonium sulfate, bacto-peptone, bacto-agar, and yeast extract were obtained from Difco Inc. Reagent grade *N*,2,6-trichloro-4-benzoquinone imine, boron trifluoride etherate, 4-hydroxybenzoic acid (*p*HBA), and phosphomolybdic acid were obtained from Eastman Organic Chemical Co. Palladium on carbon (10%) was obtained from Matheson Coleman and Bell. Protocatechuic acid and L-methionine were obtained from Sigma Chemical Co. Solanesol, ubiquinone-6, and ubiquinone-9 were generous gifts of Dr. W. Wiss of Hoffmann-La Roche, Ltd., Basel, Switzerland. All chemicals and solvents used in these studies were at least certified ACS grade. Phytol and deuterated chloroform (99.8 atom % deuterium) were obtained from the Aldrich Chemical Co. 4-Hydroxy-[U-^{14}C]benzoic acid (435 mCi/mmol) with radiopurity

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[1] Abbreviations used: *p*HBA, 4-hydroxybenzoic acid; TLC, thin-layer chromatography; 3,4-DHBA, 3,4-dihydroxybenzoic acid; Q_6, ubiquinone-6; 3,4-DHHB, 3,4-dihydroxy-5-hexaprenylbenzoic acid; 3,4-DHNB, 3,4-dihydroxy-5-nonaprenylbenzoic acid; 3,4-DHPB, 3,4-dihydroxy-5-phytylbenzoic acid.

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>99.9% was prepared by sodium fusion of DL-[U-^{14}C]tyrosine (New England Nuclear) according to Ho et al. (1973). 4-Hydroxy[*carboxy*-^{14}C]benzoic acid (53 mCi/mmol) was prepared from 4-benzyloxy[*carboxy*-^{14}C]benzoic acid (custom synthesis by Amersham-Searle) by a reduction with H_2 in the presence of 10% palladium on carbon at atmospheric pressure. The product was purified to >99.9% radiopurity by TLC on silica gel G (Brinkmann) (R_f = 0.53) with the solvent system benzene–methanol–acetic acid (45:4:2). 4-Hydroxy[U-^{3}H]-benzoic acid (572 mCi/mmol) was custom synthesized by using catalytic exchange by Amersham-Searle Corp. under the direction of Dr. Bryan W. Baker. The product was purified by multiple TLC on silica gel G with a solvent mixture of benzene–dioxane–acetic acid (90:25:4). The final product was >99.9% radiochemically pure. L-[*methyl*-^{3}H]Methionine (230 mCi/mmol) was obtained from New England Nuclear Co.

Chromatographic Materials. Unisil silica gel G (100–200 mesh) used for column chromatography was purchased from Clarkson Co. Silica gel G 1.0-mm preparative thin-layer plates were obtained from Analabs, Inc. Silica gel G 0.25-mm analytical thin-layer plates, with and without F-254 fluorescent indicator, were obtained from the Brinkmann Instrument Co.

Saccharomyces cerevisiae Strains. Ubiquinone-deficient strain E3-24 was a gift from Dr. Alexander Tzagoloff (Tzagoloff et al., 1975). It was derived from parent strain D273-10B (Sherman et al., 1968) by mutagenesis with ethyl methylsulfonate. Isolated mitochondria from E3-24 exhibited comparable electron-transport activities to strain D273-10B when reconstituted with ubiquinone-2 (Tzagoloff et al., 1975). Strain X963-18C (*amet2*) was kindly supplied by The Yeast Genetic Stock Center at the University of California, Berkeley. All strains were maintained on YPAD slants (1% yeast extract, 2% bacto-peptone, 0.003% adenine sulfate, 2% dextrose, and 2% bacto-agar) and examined for auxotrophic markers prior to experimental use.

Culture Media. E3-24 was routinely grown in SD media composed of 0.67% yeast nitrogen base and 2% dextrose. X963-18B was grown on a minimal media designated SDAH + Met composed of 0.67% yeast nitrogen base, 2% dextrose, 0.003% adenine sulfate, 50 μg/mL L-histidine, and 50 μg/mL L-methionine. Deletion of methionine produces SDAD media.

Analytical Methods. 3,4-Dihydroxy-5-poly(prenyl)benzoic acid was quantitated by its absorption at λ_{ether}^{max} = 253 nm (E = 2500) with a Zeiss PMQ II spectrophotometer. Ultraviolet scanning spectra were obtained by using a Beckman DB-GT spectrophotometer. Nuclear magnetic resonance spectra were obtained with a Varian HA 100 NMR spectrometer. Radioactivity was quantitated by using Bray's cocktail (Bray, 1960) in a Packard Model 4322 liquid scintillation spectrophotometer.

Mass spectra were obtained by direct probe with an LKB 9000 computer interfaced GLC–MS. Mass spectra were obtained at various electron impact voltages and different temperatures. Field desorption mass spectra were obtained with a Varian MAT731 high-resolution, double-focusing Maltaeich-Herzog geometry mass spectrometer (courtesy of Dr. Rinehart, University of Illinois, Urbana).

Extraction of Ubiquinone and Biosynthetic Intermediates from Yeast. A quantitative method for the extraction of ubiquinone and its biosynthetic intermediates from *Saccharomyces cerevisiae* was developed. Previous procedures (Gordon & Stewart, 1969) employed both heat and alkali to directly saponify yeast cells in order to extract efficiently ubiquinone and related compounds. These conditions, however, are favorable to base-catalyzed aromatic decarboxylation with subsequent polymerization of poly(prenyl)phenols or catecholic-type intermediates. In view of these possibilities, a milder but as efficient extraction procedure was devised to protect against chemical decomposition. Harvested yeast cells were first washed with distilled H_2O and then lyophilized. Weighed pellets were finely ground and extracted by stirring for 12 h at 4 °C with 100 mL of acetone per g dry weight of cells, after which an equal volume of pure anhydrous diethyl ether was added and the mixture allowed to extract for an additional 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, and then to a viscous lipid under a stream of N_2. The radioactive extracts were analyzed by TLC with 2 × 20 cm Brinkmann Sil-G (0.25-mm) TLC plates developed for 15 cm. Radioactive areas on the TLC plate were detected with a Packard radiochromatographic scanner, Model 7201.

Table I: TLC R_f Values[a] of Ubiquinone and Ubiquinone Biosynthetic Intermediates

compound	solvent system[b] I	II	III	IV	V
*p*HBA	0.40	0.10	0.00	0.00	0.18
3,4-DHPB	0.75	0.70	0.23	0.20	0.86
3,4-DHHB	0.67	0.65	0.20	0.14	0.72
3,4-DHNB	0.67	0.65	0.20	0.14	0.72
4-hydroxy-5-nonaprenyl-benzoic acid	0.73	0.71	0.27	0.21	0.81
ubiquinone-6	0.82	0.92	0.61	0.53	SF[c]
ubiquinone-9	0.82	0.92	0.61	0.53	SF

[a] Silica gel G (5 × 20 cm; 250 μm) (Brinkmann) analytical TLC plates were developed 15 cm in the solvent systems. [b] I, benzene-acetone (1:1); II, benzene–chloroform (7:1); III, cyclohexane–diethyl ether–acetic acid (50:50:0.5); IV, cyclohexane–diethyl ether (8:2); V, chloroform–methanol–acetone (10:3:20). [c] SF = migration at solvent front.

Synthesis and Isolation of 3,4-Dihydroxy-5-nonaprenylbenzoic Acid and 3,4-Dihydroxy-5-phytylbenzoic Acid. 3,4-Dihydroxy-5-nonaprenylbenzoic acid and 3,4-dihydroxy-5-phytylbenzoic acid were prepared according to the method of Daves et al. (1967) by using twice-recrystallized or distilled reagents. Strict adherence to anhydrous reaction conditions was critical for a successful synthesis. The products were isolated and purified to >99% homogeneity by multiple preparative TLC with the various combinations of solvent systems described in Table I. The product yields were low, ranging from 1.5 to 5% for this reaction.

Results

Identification of a Novel Polar Intermediate in Lipid Extracts of E3-24. Saccharomyces cerevisiae strain E3-24 is unable to synthesize ubiquinone-6. When 1 μCi of [U-^{14}C]-*p*HBA was added to an early exponential (20 Klett units) culture of E3-24 in YPD medium and harvested at early stationary phase, radioactivity from *p*HBA was found to accumulate in the acetone–ether lipid extract. TLC analysis of this lipid extract revealed a single peak of radioactivity chromatographing at R_f = 0.67, 0.65, 0.20, and 0.14 in solvent systems I, II, III, and IV, respectively (Table I; Goewert et al., 1977). This benzoate-type compound was found to be more polar in every solvent system examined than the most polar benzoate-type intermediate previously identified, i.e., 4-hydroxy-5-poly(prenyl)benzoic acid (Table I). Further examination found this compound to give a positive reaction for Gibbs reagent (Whistance & Threlfall, 1967), indicating this

compound to be phenolic in nature. When isolated from the TLC plate and rechromatographed, approximately 30–40% of this labeled compound was observed to break down and give rise to a less polar nonradioactive species whose relative mobilities are only slightly more polar than those of 2-octaprenylphenol, the first decarboxylated pathway intermediate found in bacteria. When [U-^{14}C]*p*HBA was used to label cultures of E3-24, this aforementioned breakdown product was found to contain radioactivity, indicating its origin results from the decarboxylation of the more polar compound accumulated in E3-24.

An identical experiment was performed as before except with [*carboxy*-^{14}C]*p*HBA as the labeled precursor. TLC analysis of the acetone–ether lipid extract produced similar results. The major peak of radioactivity again migrated at R_f = 0.20 in solvent system III (Table I). This band was isolated from the silica gel and sequentially chromatographed in solvent systems IV and II, demonstrating R_f values identical with that found for the major radioactive peak labeled by [U-^{14}C]*p*HBA. Therefore, this major radioactive band contains a carboxyl moiety. The minor peak migrating at R_f = 0.65 was not radioactive, indicating the absence of a carboxyl function present in [*carboxy*-^{14}C]*p*HBA.

UV spectra were obtained for the labeled compound and its decarboxylation product, revealing striking similarity to spectra of 3,4-dihydroxybenzoic acid and 1,2-dihydroxybenzene (figure not shown).

Isolation of 3,4-Dihydroxy-5-hexaprenylbenzoic Acid from E3-24. In order to pursue structural studies of this new metabolite, its large-scale isolation was undertaken. A 900-mg (2.6×10^5 cpm) sample of lipid extract was obtained from 43 L of E3-24 grown on SD media in the presence of [G-^{3}H]-*p*HBA (1 μCi/L). The lipid extract was redissolved in petroleum ether and charged to a 2 × 20 cm alumina column, equilibrated with petroleum ether. A step gradient of 0.5% increases of diethyl ether in 250 mL eluted 9.3 mg of material containing 1.12×10^5 cpm in the 2.5% ether fraction. This material was next redissolved in diethyl ether and applied equally to two 20 × 20 cm, 1-mm Sil-G preparative TLC plates and developed in solvent system I. The 2.2 mg of recovered material containing 8.85×10^4 cpm was next chromatographed in solvent system V, yielding 200 μg of compound containing 5.02×10^4 cpm. A final TLC purification step reusing solvent system I produced a total of 60 μg of pure compound of a specific activity of 5.41×10^5 cpm/mg (Goewert et al., 1977).

Disappearance of the labile carboxyl-containing species, primarily via decarboxylation, was observed throughout this purification scheme, resulting in the loss of approximately 95% of the starting compound. Therefore, the direct analysis of this compound by techniques other than mass spectrometry was impossible. Since the structures for demethoxy- and demethylubiquinone, and other intermediates in ubiquinone biosynthesis present at low endogenous levels in the rat (Houser & Olson, 1977), were elucidated by comparison to synthetic homologues, a similar approach was employed in this investigation. Nonaprenyl homologues were synthesized, and their chemical and spectral properties were compared to those of the new intermediate.

Identification of 3,4-Dihydroxy-5-hexaprenylbenzoic Acid. In view of this compound's increased polarity as compared to that of 4-hydroxy-5-poly(prenyl)benzoate, similarity of ultraviolet spectra as compared to those of 3,4-dihydroxybenzoate, and reasonable structure as suggested by pathway intermediates past the block, a likely candidate as to its structure would be 3,4-dihydroxy-5-hexaprenylbenzoate (3,4-DHHB). The nonaprenyl homologue 3,4-dihydroxy-5-nonaprenylbenzoic acid (3,4-DHNB) was synthesized and purified by the scheme used to purify the natural product, and its chemical and spectral properties were compared. 3,4-DHNB was found to comigrate with the natural product from E3-24 in all straight-phase TLC solvent systems (Table I). Polyprenyl homologues containing six to nine isoprene units are known to be inseparable by straight-phase chromatography. 3,4-DNBA on TLC plates demonstrated a positive reaction for Gibbs reagents. Furthermore, the UV spectra of 3,4-DHNB were found to be identical with those of the natural product from E3-24. Similarly, 3,4-DHNB was found to be unstable, readily breaking down by decarboxylation to a compound which comigrated in straight-phase TLC systems with the breakdown product from the natural product. Furthermore, 3,4-DHNB had a UV spectrum identical with that found for E3-24's breakdown product.

A criterion commonly used to establish structural homology between compounds containing isoprenyl chains of various length is mass spectrometry. Mass spectra of structural homologues share a series of ions generated by fragmentation of the nonisoprene moiety (Muraca et al., 1967). To gain additional insight into the mass spectral fragments produced by the 3,4-dihydroxybenzoate nucleus, alkylated at the 5 position, we synthesized 3,4-dihydroxy-5-phytylbenzoic acid (3,4-DHPB).

Mass spectra obtained by direct inlet for 3,4-DHHB, 3,4-DHNB, and 3,4-DHPB are shown in Figure 1. All three spectra demonstrated identical fragments with regard to certain diagnostic ion fragments. For example, in 3,4-DHHB, as in the others, the unstable tropylium ion with *m/e* 167 yielded a very prominent *m/e* 123 peak upon decarboxylation. The catecholic tropylium characteristically gave up CO to yield *m/e* 95, also a prominent ion in all spectra. By a similar mechanism, the chromanylium ion, *m/e* 207, generated by cyclization of the side chain at the 5 position, readily decarboxylated to yield an ion at *m/e* 163. A weak molecular ion agreeing with the calculated molecular weight was found for 3,4-DHHB and 3,4-DHPB at *m/e* 562 (0.5%) and *m/e* 432 (0.9%). In addition, fragments resulting from decarboxylation of the molecular ion are observed at *m/e* 518 for 3,4-DHHB and *m/e* 388 for 3,4-DHPB.

A molecular ion for 3,4-DHNB was not obtained by direct inlet due to its thermal lability. High-resolution field desorption mass spectrometry was employed to circumvent the compound's unstable nature. A molecular ion was identified (Figure 2) at *m/e* 765 ± 1 for 3,4-DHNB. The observed *m/e* for the molecular ion agrees with the calculated molecular weight for this compound. Furthermore, the decarboxylated species corresponding to $M^+ - 44$ is present at *m/e* 721.

The nuclear magnetic resonance spectral assignments of 3,4-DHNB and 3,4-dihydroxybenzoic acid are presented in Table II. The spectral assignments are consistent with the assigned structure of the compound. The prenyl side-chain protons agree well with those identified for other ubiquinone precursors (Morton, 1965). The carboxy group and aromatic protons are shifted slightly upfield from the analogous protons of 3,4-dihydroxybenzoic acid. Furthermore, a singlet at 7.5 ppm demonstrates the absence of the 5-hydrogen of protocatechuic acid, indicating ring substitution at this position. In addition, the inductive effect of the side chain deshields the 2-hydrogen, shifting its resonance frequency 0.4 ppm, possessing a spin–spin coupling (J = 16 Hz) constant of the same order as that of 3,4-dihydroxybenzoic acid. On the basis of

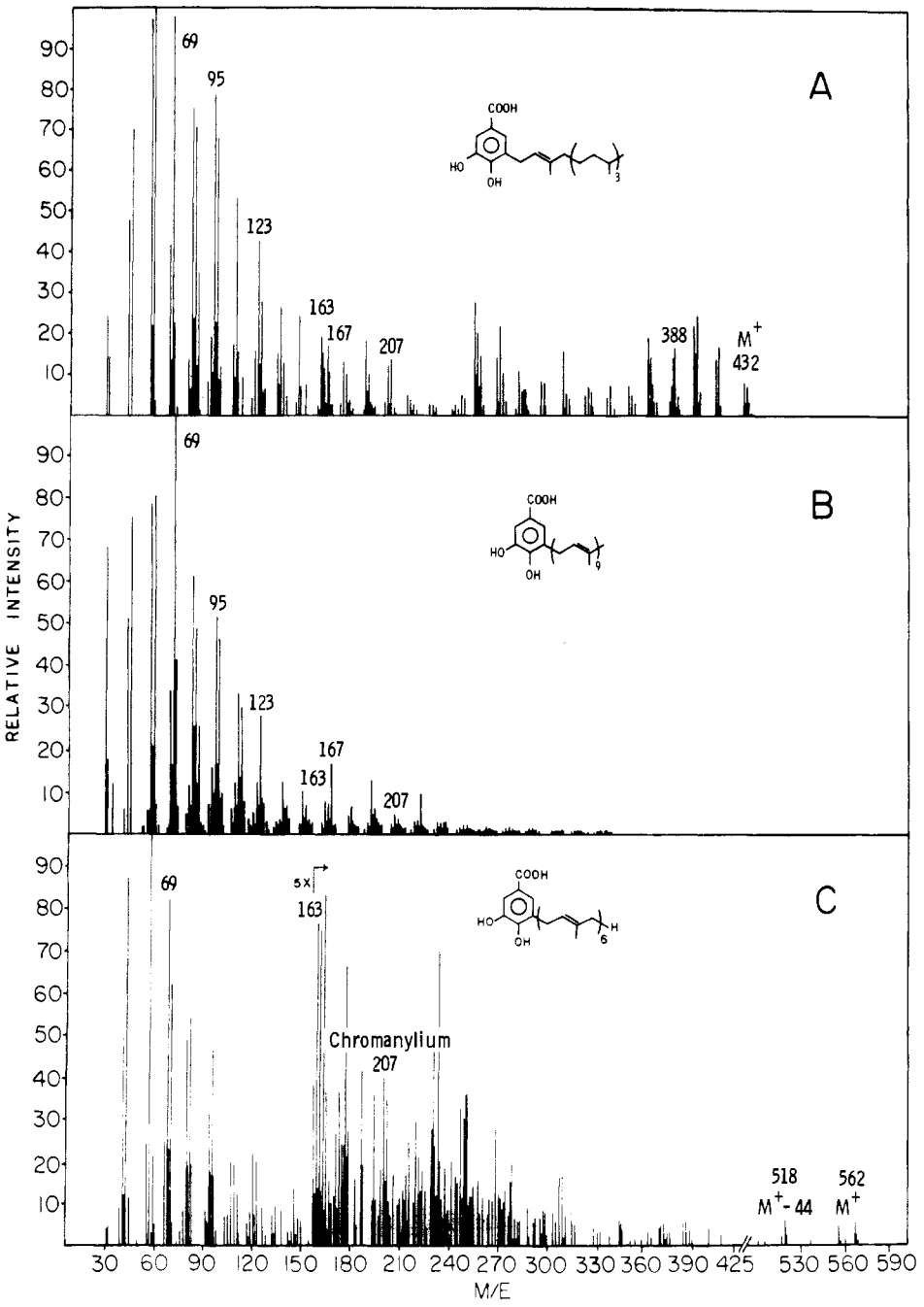

FIGURE 1: Medium-resolution mass spectra of homologous alkylated protocatechine acids obtained by direct inlet technique. (A) 3,4-Dihydroxy-5-phytylbenzoic acid; (B) 3,4-dihydroxy-5-nonaprenylbenzoic acid; (C) 3,4-dihydroxy-5-hexaprenylbenzoic acid.

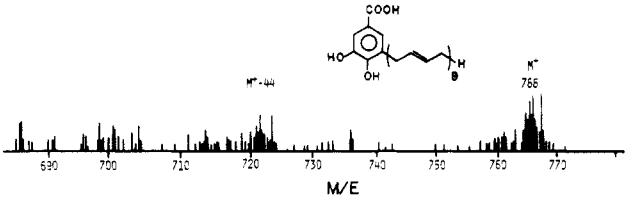

FIGURE 2: High-resolution field desorption mass spectra of 3,4-dihydroxy-5-nonaprenylbenzoic acid.

the accumulated compositive evidence from mass spectral data, comigration in TLC, isotopic labeling, and identical chemical lability, the compound accumulated in E3-24 is 3,4-dihydroxy-5-hexaprenylbenzoic acid.

In Vivo Conversion of 3,4-Dihydroxy-5-hexaprenylbenzoate by met2 Mutant. To demonstrate that 3,4-DHHB is a bona fide intermediate in the biosynthesis of ubiquinone-6 in yeast, it was necessary to show an in vivo precursor–product relationship. It was found that *S. cerevisiae* mutants blocked in methionine synthesis accumulated 3,4-DHHB when deprived of methionine. The rationale was that methionine deprivation in these strains would stop the synthesis of *S*-adenosylmethionine and hence interrupt ubiquinone biosynthesis at steps prior to methylation reactions. When methionine was removed from SDAH + Met media of a mid-log culture of strain X963-18C (genotype *αmet2*, *his8*, *ade8*) blocked in methionine biosynthesis at homoserine *O*-transacetylase (EC 2.3.1.31) (Cherest et al., 1971), reactions requiring *S*-adenosylmethionine as a substrate are rapidly inhibited. During methionine starvation, radioactivity from exogenously added [U-^{14}C]*p*HBA and [*carboxy*-^{14}C]*p*HBA was observed to accumulate into a compound identified as 3,4-DHHB, based on TLC comigration with 3,4-DHHB, ultraviolet absorption spectra identical with those of 3,4-DHNB and 3,4-DHHB, and mass spectra identical with that found for 3,4-DHHB isolated

Table II: NMR Assignments for 3,4-Dihydroxy-5-nonaprenylbenzoic Acid and Protocatechuic Acid[a]

ppm			
protocatechuic acid	3,4-DHNB	protons	assignment
	1.63 (s)	24	trans methyl of side chain
	1.68 (s)	3	cis methyl of side chain
	1.73 (s)	3	trans methyl of first isoprene group
	2.03 (m)	32	side-chain methylene
	4.10 (d)	2	methylene adjacent to ring
3.58 (s)	3.75 (s)	2	hydroxyl protons
	5.10 (m)	8	side-chain methine
	5.22 (t)	1	methine of first isoprene
6.85 (d)		1	aromatic proton meta to carboxyl group
	7.20 (s)	1	aromatic proton ortho to carboxyl and hydroxyl groups
	7.28 (m)	1	aromatic proton ortho to side chain
7.50 (d)		1	aromatic proton ortho to carboxyl and aromatic groups
7.53 (s)		1	aromatic proton ortho to carboxyl and hydroxyl groups

[a] Chemical-shift values for protocatechuic acid were obtained from literature sources; s = singlet, d = doublet, t = triplet, and m = complex splitting pattern.

from E3-24. Three identical cultures of X963-18B in SDAH media, minus methionine, were labeled for 3 h with labeled *p*HBA. Cultures A and B were labeled with [U-^{14}C]*p*HBA while culture C was labeled with [*carboxy*-^{14}C]*p*HBA. Label from both types of isotopic *p*HBA was observed to accumulate in 3,4-DHHB during methionine deprivation. In control experiments where [U-^{14}C]*p*HBA was added to aerobically growing cultures of *met2* in SDAH + Met medium, 3,4-DHHB was not detected. Moreover, the label was found to accumulate solely into ubiquinone-6. Therefore, 3,4-DHHB accumulates only in the absence of methionine.

Radioactive *p*HBA was next washed from the three methionine-9-starved *met2* cultures, and one labeled culture was resuspended in SDAH + methionine (cold) while the remaining two were resuspended in SDAH + [*methyl*-^{3}H]-methionine. The methionine level was identical in all tubes (50 μg/mL). Logarithmic growth resumed in all cultures upon the readdition of methionine. Samples from each exponentially growing *met2* culture were taken every hour following the readdition of methionine, and the fate of the accumulated ^{14}C-labeled 3,4-DHBA was followed in the presence of the [*methyl*-^{3}H]methionine. As can be seen in Figure 3, the accumulated [*ring*-^{14}C]3,4-DHNB (derived from [U-^{14}C]-*p*HBA-labeled cultures) and [*carboxy*-^{14}C]3,4-DHHB were rapidly metabolized upon the readdition of methionine. This event is paralleled by a resumption of ubiquinone biosynthesis, as indicated by the incorporation of [^{3}H]methyl groups into ubiquinone (Figure 3B,C). A precursor–product relationship for 3,4-DHHB and ubiquinone-6, respectively, is demonstrated in panels A and B by noting the reciprocal relation between incorporation of ^{14}C into ubiquinone-6 and disappearance of [*ring*-^{14}C]3,4-DHHB.

The rates of disappearance of [*ring*-^{14}C]3,4-DHHB and [*carboxy*-^{14}C]3,4-DHHB are essentially the same (Figure 3A,B). During the first hour of resumed logarithmic growth

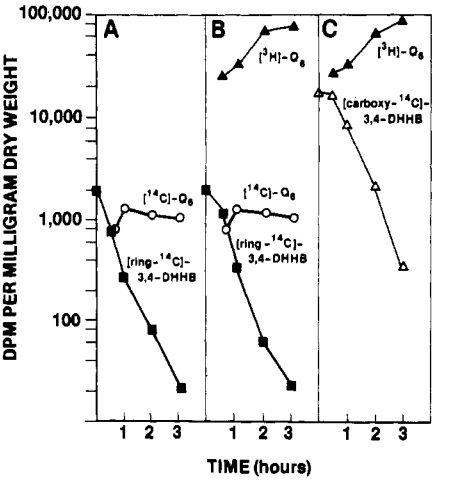

FIGURE 3: Fate of 3,4-dihydroxy-5-hexaprenyl[^{14}C]benzoic acid upon readdition of L-methionine. Aliquots of 34 mL of cultures A and B were prelabeled with 3 μCi of [U-^{14}C]*p*HBA, and C was prelabeled with [*carboxy*-^{14}C]*p*HBA. The degree of labeling of 3,4-DHHB is shown at zero time. Each of the *met2* cultures prelabeled with isotopic *p*HBA was washed twice with SDAH medium to remove isotopic precursor. Cultures B and C were resuspended in SDAH + 100 μCi of [*methyl*-^{3}H]methionine while culture A was resuspended in SDAH containing unlabeled methionine. The three cultures were then grown in the presence of labeled or unlabeled methionine in a gyrorotatory bath at 31 °C. Samples (5 mL) from each culture were taken at 0.5, 1.0, 2.0, and 3.0 h. The samples were extracted and analyzed by TLC.

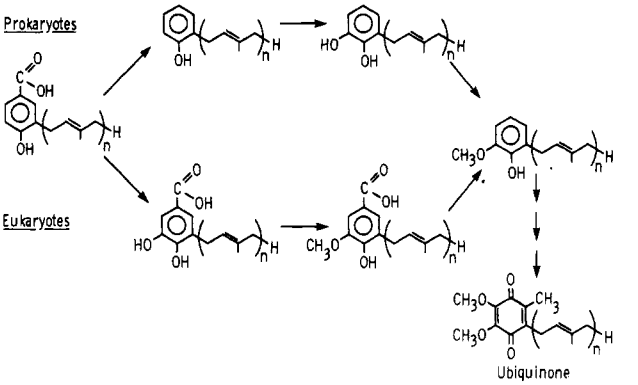

FIGURE 4: Divergent and common pathways for ubiquinone biosynthesis in eukaryotic and prokaryotic organisms.

and ubiquinone biosynthesis, the disappearance of [*ring*-^{14}C]3,4-DHHB is quantitatively accounted for by the incorporation of ^{14}C into ubiquinone-6. Three hours after the readdition of methionine, the incorporation rate of ^{14}C into ubiquinone-6 from [*ring*-^{14}C]3,4-DHHB remained high but appeared diminished as plotted per milligram of yeast, because of the rapid growth and endogenous synthesis of 3,4-DHHB. As the carboxyl-labeled 3,4-DHHB disappeared, [^{3}H]methyl groups from methionine appeared in otherwise unlabeled Q_6. These data establish a precursor–product relationship between 3,4-DHHB and Q_6.

Discussion

The results of this study provide evidence that the pathway for the biosynthesis of ubiquinone in the eukaryote *Saccharomyces cerevisiae* is different from that described for the prokaryotes *Escherichia coli* (Gibson, 1973) and *Rhodospirillium rubrum* (Friis et al., 1966). The identification of 3,4-dihydroxy-5-hexaprenylbenzoate in yeast demonstrates that C-3 hydroxylation of 4-hydroxy-3-hexaprenylbenzoate to form a catechol occurs prior to decarboxylation. Nowicki et al. (1972) first proposed a pathway for the biosynthesis of ubiquinone-9 in the rat which differed from the pathway estab-

lished for *E. coli* (Figure 4). This pathway was proposed to explain several anomalous observations in the synthesis of ubiquinone-9 in the rat and predicted the occurrence of a protocatechuate-type intermediate. Enzymatic decarboxylation of 3-poly(prenyl)-4-hydroxybenzoate to form 2-poly(prenyl)phenol, as described in *E. coli*, did not appear to occur in the rat. Furthermore, workers in this laboratory were unable to detect 2-nonaprenylphenol in lipid extracts from rat liver, kidney, or yeast under experimental conditions which permitted the isolation of other intermediates at levels as low as 0.1 μg/g liver (Nowicki et al., 1972). Likewise, Parson & Rudney (1965) and Nilsson et al. (1968) were unable to detect 2-nonaprenylphenol in rat or mouse tissues. In addition, radiolabeled synthetic 2-nonaprenylphenol was not incorporated into ubiquinone with either liver slices or isolated, perfused liver. Furthermore, no dilution in ubiquinone's specific activity could be detected. Finally, Gardner, in this laboratory,[2] prepared 3-nonaprenyl-4-hydroxy[*carboxy*-^{14}C]benzoic acid and observed no enzymatic decarboxylation upon incubation with tissue slices, mitochondria, or inner membrane fragments of mitochondria. Taken together, these observations support the hypothesis that in the rat, and perhaps in other eukaryotic species, a pathway distinct from that in *E. coli* exists which delays decarboxylation to a point beyond 4-hydroxy-3-poly(prenyl)benzoate.

Of major importance to the success of this study was the availability of ubiquinone-deficient mutants of *Saccharomyces cerevisiae* (Tzagoloff et al., 1975), permitting studies to be performed by using a simple unicellular eukaryote in a fashion similar to that of the *E. coli*. Strain E3-24, when incubated with [U-^{14}C]*p*HBA, was found to accumulate in a novel polar lipid-type compound identified as 3,4-dihydroxy-5-hexaprenylbenzoate. The ultraviolet spectra of the spontaneous breakdown product of 3,4-DHPB closely resembled those of catechol. Furthermore, this breakdown product contained no radioactivity when labeled with [*carboxy*-^{14}C]*p*HBA in vitro. This breakdown product was observed to accumulate only when using [U-^{14}C]*p*HBA to label E3-24 and to be chemically generated by the decarboxylation of 3,4-DHHB with 1 N HCl. 3,4-Dihydroxy-5-nonaprenylbenzoate was found to undergo a similar decarboxylation, generating the homologous substituted catechol. This catechol (2-hydroxy-5-hexaprenylphenol) was not converted to ubiquinone-6 in yeast mitochondria. These observations indicate that in yeast, and possibly in other eukaryotes, 3-poly(prenyl)catechol is not a normal intermediate in ubiquinone synthesis. This is unlike *E. coli*, in which 3-octaprenylcatechol is presumed to be a normal intermediate (Gibson, 1973). It is also of interest that the *E. coli* mutant (*ubi*D) devoid of 3-octaprenyl-4-hydroxybenzoate decarboxylase (Cox et al., 1969) retained an alternate pathway accounting for 20% of ubiquinone synthesis. It is possible that this alternate pathway in *E. coli* is identical with the one which becomes dominant in eukaryotic organisms. The success observed in this study in isolating ubiquinone-6 is attributable in part to the gentle extraction procedure devised to prevent the chemical destruction and production of artifacts from ubiquinone intermediates (Nowicki et al., 1972). Nyns et al. (1968) carried out systematic experiments aimed at establishing the most efficient method of extracting lipids from *Candida lipolytica*. Acetone–diethyl ether (1:1) was found to be most effective for extraction of the neutral lipid fraction. A variation of the Nyns' procedure was necessary to obtain an optimal extraction efficiency for ubiquinone as ascertained by greater than 90% by the recovery of tracer quantities of exogenously added ubiquinone to a lyophilized yeast pellet prior to extraction. Extraction of [U-^{14}C]*p*HBA-labeled E3-24 after saponification resulted in a single radioactive peak remaining at the origin, even in the most polar TLC solvent systems.

In view of the particularly labile nature of 3,4-dihydroxy-5-hexaprenylbenzoate, it was impossible to isolate milligram quantities of natural product. Therefore, structural identity was demonstrated by comparison with a synthetic homologue, 3,4-dihydroxy-5-nonaprenylbenzoate (DHNB), and its synthetic analogue, 3,4-dihydroxy-5-phytylbenzoate (DHPB). The nonaprenyl homologue demonstrated identical chromatographic behavior and elicited the expected positive reactions to detection sprays, when compared to the natural homologue from E3-24. The mass spectra for the hexaprenyl and nonaprenyl compounds demonstrated homologous spectra and rendered the predicted molecular ions at *m/e* 562 and 765 ± 1, respectively, with the diagnostic chromanylium and trophylium ions present at *m/e* 207 and 167, respectively.

When exploring the possibility of using methionine biosynthetic mutants to study the points of methylation in ubiquinone-6 biosynthesis in yeast, we observed a strain blocked in methionine biosynthesis at homoserine acetyltransferase (Cherest et al., 1971) to accumulate 3,4-dihydroxy-5-hexaprenylbenzoate when starved for methionine in the presence of [U-^{14}C]- or [*carboxy*-^{14}C]*p*HBA. The addition of [*methyl*-^{3}H]methionine to the starved cultures reinitiated normal growth and ubiquinone-6 biosynthesis. When [*ring*-^{14}C]3,4-DHHB was present, there was a stoichiometric incorporation of label into ubiquinone-6 during the first hour of resumed ubiquinone biosynthesis. When [*methyl*-^{3}H]methionine was added, the incorporation of counts from [*methyl*-^{3}H]-methionine into ubiquinone-6 was quantitatively proportional to the rate of synthesis of ubiquinone and inversely proportional to the loss of counts from 3,4-DHHB. A precursor–product relationship was thus established.

The accumulation of 3,4-DHHB in methionine-starved methionine auxotrophs is strong evidence that 3,4-DHHB is methylated prior to its decarboxylation. Since *S*-adenosylmethionine has already been demonstrated to be the source of both ring- and *O*-methyl groups (Guldberg & Marsden, 1975), methionine starvation should exert its action on this pathway by depleting substrate for the SAM-dependent methyltransferase reactions. This would halt ubiquinone biosynthesis effectively at the first methylation step. In fact, Goewert et al. (1978) have identified 3-methoxy-4-hydroxy-5-hexaprenylbenzoate as a new intermediate in ubiquinone-6 biosynthesis by *S. cerevisiae*.

Casey & Threlfall (1978) have shown that 5-demethoxyubiquinone-6 and ubiquinone-6 can by synthesized from 3-hexaprenyl-4-hydroxybenzoate in yeast mitochondria. The identification of 3,4-dihydroxy-5-hexaprenylbenzoic acid as an intermediate in ubiquinone-6 biosynthesis in yeast suggests that a segment of the pathway for the biosynthesis of ubiquinone in eukaryotes is divergent. The order of metabolic transformations of *p*HBA in its conversion to 6-methoxy-2-poly(prenyl)phenol in eukaryotic organisms appears to be hydroxylation, methylation, and decarboxylation.

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Solid-State Carbon-13 Nuclear Magnetic Resonance of the Lecithin Gel to Liquid-Crystalline Phase Transition†

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ABSTRACT: The temperature dependence of the ^{13}C NMR spectra of dipalmitoylphosphatidylcholine (DPPC) which has been ^{13}C labeled at the carbonyl position of the *sn*-2 chain, 2-[1-^{13}C]DPPC, is reported. In the $L_{\beta'}$ phase an axially symmetric spectrum of 112-ppm breadth is observed, and this transforms to an isotropic-like line ($\langle\Delta\sigma\rangle \sim 7$ ppm) in the L_α phase. In the intermediate P_β phase a temperature-dependent superposition of these spectra is observed, which suggests that this phase exhibits microscopic structural and dynamical properties of both the $L_{\beta'}$ and L_α phases. An analysis of the spectral line shapes leads to the conclusion that the appearance of the isotropic-like line in the $P_{\beta'}$ phase is primarily due to a conformational change at the *sn*-2 carbonyl which is complete at the main transition. Increased rates of axial diffusion in the $P_{\beta'}$ phase may contribute to the narrowing.

Phase equilibria in the binary lecithin–water system have been the subject of a number of investigations using a variety of structural, spectroscopic, and thermodynamic methods. For aqueous dispersions of dimyristoyl- and dipalmitoylphosphatidylcholine (DMPC and DPPC, respectively), calorimetric measurements identify two endothermic transitions, a broad, low enthalpy pretransition [T_c' = 35.3 °C (DPPC)] and a sharp, high enthalpy main transition [T_c = 41.3 °C (DPPC)] (Chapman et al., 1967). With respect to these different lipid phases, X-ray scattering patterns obtained from unoriented samples have identified the lattice types and dimensions and the general low resolution features of the acyl chain conformations (Tardieu et al., 1973; Janiak et al., 1976, 1979). At temperatures below the pretransition, the lipid molecules are arranged in a one-dimensional, lamellar $L_{\beta'}$ phase in which the acyl chains are predominantly extended (all-trans conformation) and are assumed to be tilted at 30° with respect to the bilayer normal to accommodate the bilayer thickness of 47 Å. For dispersions in excess water, as studied

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