THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 24, Issue of December 25, pp. 11802-11806, 1980
Printed in U.S.A.

Identification of Deoxy-α-tocopherolquinol as Another Endogenous Electron Donor for Biohydrogenation*

(Received for publication, August 1, 1980)

Peter E. Hughes and Samuel B. Tove‡

From the Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27650

Solvent extracts of *Butyrivibrio fibrisolvens* contain 2-[3, 7, 11, 15-tetramethylhexadecyl]-3, 5, 6-trimethylbenzoquinol (deoxy-α-tocopherolquinol) that can serve as an alternate electron donor for α-tocopherolquinol for the biohydrogenation of *cis*-9,*trans*-11-octadecadienoate. In addition, the cell extracts contain deoxy-α-tocopherolquinone. This compound arises metabolically from α-tocopherolquinone via dehydration to trimethylphytylbenzoquinone followed by hydrogenation to deoxy-α-tocopherolquinone. Although the hydrogenation of the isoprene double bond and the conjugated fatty acid both use NADH as the primary reductant, the two reactions appear to be catalyzed by different enzymes.

The anaerobic rumen bacterium *Butyrivibrio fibrisolvens* carries out the biohydrogenation of linoleic acid in a two-step process. The first step is the isomerization of linoleic acid to *cis*-9,*trans*-11-octadecadienoate, and the second is the hydrogenation of this conjugated acid to produce *trans*-11-octadecenoate (1). Although the reducing hydrogens could come from water (2), the source of the 2 electrons involved in the reduction could come from NADH, reduced methyl viologen, and an uncharacterized endogenous electron donor(s) (3).

We have recently shown that a chloroform extract of the cell extract of *B. fibrisolvens* possessed electron donor activity in the standard reduction assay (4). When the chloroform extract was chromatographed on thin layer plates, four fluorescent spots were found to have donor activity in the presence of sodium borohydride. The fastest migrating spot could be resolved into two by chromatography in the other direction with pentane. Two of these were identified as α-tocopherolquinone and α-tocopherolquinol (4). In this paper, we report the identification of the compound migrating with pentane as deoxy-α-tocopherolquinone and another spot (R_F 0.57) as deoxy-α-tocopherolquinol.

EXPERIMENTAL PROCEDURES[1]

Materials

Methyl viologen dichloride, sodium dithionite, sodium borohydride, and NADH were obtained from Sigma. Authentic α-tocopherolquinone, trimethylphytylbenzoquinone, and [3,4-^{14}C]-α-tocopherol were the generous gifts of Hoffmann-La Roche. The ^{14}C-α-tocopherolquinone was prepared from ^{14}C-α-tocopherol as previously described (5). The D_2O (99%) was purchased from Stohler Isotope Chemicals. Solutions of the quinones or hydroquinones were stored in ampules sealed under oxygen-free nitrogen in a light-tight container at -20°C. Methyl cis-9,trans-11-octadecadienoate was prepared by reduction of methyl trans-11-octadecen-9-ynoate (6). The free fatty acid was obtained by hydrolysis with 2 N KOH under nitrogen. All other reagents were purchased from Fisher and, unless otherwise stated, were of analytical quality.

Methods

Anaerobic conditions were maintained throughout the isolation and assays by flushing all vessels and solutions with oxygen-free nitrogen (4) for at least 20 min. Manipulations were carried out in dim light.

B. fibrisolvens (ATCC No. 27208) was cultured and the cell extract prepared as previously described (4), as was the chloroform/ethanol (2:1) extraction and chromatography of the concentrated lipid extract. The thin layer plates were developed in acetone/toluene/methanol (2:2:1) in one direction and pentane in the second direction. Fluorescent spots were visualized at 366 nm. The lipid extract was chromatographed with a Waters Associates LC Prep 500 HPLC unit with a C-18 column using methanol/water (3:1) at a flow rate of 100 ml/min. Eluted compounds, detected by index of refraction, were collected, concentrated and then subjected to thin layer chromatography. Organic material on the plates was detected by charring with 40% H_2SO_4 at 100°C.

The fatty acid reductase preparation used was the pooled fractions of the second peak of activity from a Sepharose 6B column (3). The enzyme was stored at -20°C and prior to use was passed through an anaerobic Sephadex G-25 column with 0.1 M phosphate, pH 7.0, as the eluant. The void volume was collected and concentrated. The standard assay for cis-9,trans-11-octadecadienoate reductase activity consisted of 70 μg of enzyme protein in 0.5 ml 0.1 M phosphate, pH 7.0, containing 50 nmol of potassium cis-9,trans-11-octadecadienoate and variable amounts of an electron donor added in 10 μl of ethanol. In some cases 50 μmol of sodium dithionite or sodium borohydride was added to the reaction mixture. Incubation was carried out at 37°C for 20 min, after which the fatty acids were extracted and the unreacted, conjugated diene determined spectrophotometrically (3).

Elemental analysis was performed by Integral Microanalytical Laboratories, Inc., Raleigh, NC. High and low resolution mass spectrometry was carried out by the Research Triangle Institute, Research Triangle Park, NC. The NMR spectrometry was carried out on a Varian HA-100 spectrometer in deuterated chloroform/deuterated acetone (2:1) containing TMS as an internal standard. Samples were washed with D_2O prior to recording the NMR spectrum. Fluorescence was recorded on an SLM fluorometer and absorption spectra were recorded on a Cary Model 15 spectrophotometer.

The catalytic hydrogenation of trimethylphytylbenzoquinone was carried out according to the method of Lees (7). A flask containing 2 mg of trimethylphytylbenzoquinone and 2 mg of platinum black in 20 ml of ethanol was purged with H_2 for 3 min and then sealed. Pressure was maintained at 1520 torr for 3 h while stirring constantly at 27°C in the dark. The solution was filtered, and any hydroquinone was oxidized to the quinone by aeration for 60 sec with a Vortex mixer. This extract was then applied to thin layer plates coated with 0.5 mm of silica gel containing 5% $AgNO_3$. The plates were developed in acetone/toluene/methanol (2:2:1) and visualized by fluorescence.

Stoichiometry of Oxidation of Deoxy-α-Tocopherolquinol--The reaction mixture consisted of 140 μg of fatty acid reductase protein and 200 nmol of potassium cis-9,trans-11-octadecadienoate in 1 ml of 0.1 M phosphate, pH 7.0. Various amounts (20-200 μmol) of isolated deoxy-α-tocopherolquinol were added, and the mixture was incubated anaerobically for 60 min at 37°C. One portion of the reaction mixture was analyzed for the conjugated diene (3) and another portion extracted with chloroform in the anaerobic glove bag. For anaerobic extraction the chloroform was freed of oxygen as described (4), and for aerobic extraction it was not. The deoxy-α-tocopherolquinol and deoxy-α-tocopherolquinone were separated by thin layer chromatography and analyzed spectrophotometrically. The deoxy-α-tocopherolquinol was converted to deoxy-α-tocopherolquinone prior to spectrophotometric assay. The molar extinction coefficients were determined using purified (HPLC) synthetic compounds and were found to be 17,300 (259 nm) for deoxy-α-tocopherolquinone and 4,600 (281 nm) for the deoxy-α-tocopherolquinol in ethanol.

Stoichiometry of Reduction of Deoxy-α-Tocopherolquinone by NADH--The reaction mixture contained 37 μg of enzymic protein, 10 mg of gelatin, 300 nmol of NADH, and 3 μl of ethanol containing 30-320 nmol of deoxy-α-tocopherolquinone in 2 ml of 0.1 M phosphate, pH 7.4. The oxidation of NADH was measured spectrophotometrically at 340 nm. The amount of deoxy-α-tocopherolquinol was determined from the difference in absorption (259 nm) of an anaerobic chloroform extract before and after air oxidation.

Conversion of α-Tocopherolquinone to Deoxy-α-Tocopherolquinone--Cells from 32 liters of culture medium of B. fibrisolvens were grown and harvested as described previously (4). The cell suspension in 300 ml of anaerobic 0.1 M phosphate buffer, pH 7.0, was passed through a French pressure cell at 15,000 p.s.i., and the effluent was then centrifuged at 34,000 x g for 30 min. The supernatant was collected and 10 ml loaded onto a Sepharose 6B column (2.5 x 40 cm) made anaerobic by pre-equilibration with 1 mM methyl viologen dichloride reduced with sodium dithionite in 0.1 M phosphate buffer, pH 7.0. Fractions were collected in serum-stoppered gassed tubes and stored in anaerobic jars at -20°C. Prior to use, the enzyme fractions were passed through anaerobic Sephadex G-25 columns and the void volume collected. Each fraction was assayed for the conversion of ^{14}C-α-tocopherolquinone to ^{14}C-deoxy-α-tocopherolquinone by incubation of 10 nmol ^{14}C-α-tocopherolquinone (68,000 dpm) in 3 μl ethanol, 1.0 ml of an enzyme fraction, and 50 μmol NADH in 2 ml reaction mixture of 0.1 M phosphate buffer, pH 7.4. In experiments involving an intermediate, the same system was used without NADH. After incubation for 60 min at 37°C, the tubes were extracted with 10 ml of aerobic chloroform/ethanol (2:1) containing carrier deoxy-α-tocopherolquinone, α-tocopherolquinone, and trimethylphytylbenzoquinone. The chloroform layers were concentrated with a stream of N_2/H_2 and spotted on thin layer plates impregnated with 5% $AgNO_3$ and developed with acetone/toluene/methanol (2:2:1). Fluorescent spots corresponding to deoxy-α-tocopherolquinone (R_f 0.90), α-tocopherolquinone (R_f 0.80), and trimethylphytylbenzoquinone (R_f 0.55) were scraped off and the radioactivity determined.

Characterization of the Trimethylphytylbenzoquinone Intermediate--A combination of fractions 1, 2, and 3 containing 0.9 mg of protein were diluted to 15 ml with 0.1 M phosphate buffer, pH 7.4. After adding 50 μl of ethanol containing 50 μmol of α-tocopherolquinone, the mixture was incubated anaerobically for 60 min at 37°C and then was extracted with 150 ml of chloroform/ethanol (2:1). The chloroform layer was removed and concentrated to dryness and taken up in 100 μl pentane and then spotted on silica gel thin layer plates impregnated with

* Contribution from the Department of Biochemistry, School of Agriculture and Life Sciences and School of Physical and Mathematical Sciences. This is Paper 6536 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, North Carolina 27650. A preliminary account of this work was presented at the 71st Annual Meeting of the American Society of Biological Chemists in New Orleans, June 1980. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed.

[1] The "Experimental Procedures" are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014. Request Document No. 80M-1606, cite authors, and include a check or money order for $1.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

5% $AgNO_3$. The fluorescent spot at R_f 0.55 was scraped and the compound eluted with 2 ml pentane. Separate solutions of the compound and trimethylphytylbenzoquinone in 2 ml of pentane were deuterated at the same time via a "Y" tube with deuterium gas at 760 torr in the presence of 2 mg platinum black for 1.5 h. The solutions were filtered and chromatographed on thin layer plates impregnated with 5% $AgNO_3$. The yellow fluorescent spot at R_f 0.9 was scraped, eluted with 2 ml pentane, and analyzed for isotopic enrichment by mass spectrometry.

RESULTS

The substance migrating at R_F 0.57 (Fig. 1, Ref. 4) in the thin layer system was isolated in milligram quantities by preparative high pressure liquid chromatography. The refractive index detector pattern showed an isolated, single, symmetrical peak at 1.85 void volumes. When concentrated, this isolated substance was active in the electron donor enzyme assay system without the addition of sodium borohydride or dithionite. When chromatographed, it gave a single yellow fluorescent spot at R_F 0.57. Acid charring of the plate resolved no other spots. Thus, the isolated compound was presumed to be pure and in the reduced form.

Characterization of the Endogenous Donor—The absorption spectrum in absolute ethanol showed a shoulder at 285 nm with no valley (Fig. 1*A*). When oxidized by shaking with air, the spectrum changed, giving a maximum at 259 nm and a shoulder at 268 nm. In a chloroform/ethanol solvent system, the oxidized compound gave a spectrum showing fine structure with peaks at 262 and 268 nm (Fig. 1*B*). The fluorescence spectrum of the air-oxidized compound, when excited at 330 nm, had peaks at 366 and 389 nm and a minimum at 375 nm (Fig. 1*C*). Overall, these spectra resembled the spectra of α-tocopherolquinone (4, 8); hence, it was presumed to be a benzoquinone.

Elemental analysis showed the absence of sulfur, nitrogen, and phosphorus. High resolution mass spectrometry showed it to have the empirical formula:

$C_{29}H_{50}O_2$

Nuclidic mass observed: 430.3793
Calculated: 430.3810

Proton NMR spectra of the oxidized form of the isolated compound gave no signal at 6.5 to 7.0 ppm, demonstrating the absence of hydrogens on a quinone ring. Signals seen were as follows: (*a*) a doublet centered at 0.92 ppm (J = 4 Hz) integrating for 15 hydrogens, consistent with protons of five methyl groups on an aliphatic chain; (*b*) three singlets at 2.02 ppm, 2.14 ppm, and 2.19 ppm which integrated for nine protons, consistent with three methyl groups attached to a quinone ring; and (*c*) a triplet centered at 2.62 ppm (J = 4 Hz) integrating for two protons, consistent with a methylene group attached to a quinone ring. The rest of the spectrum consisted of a broad multiplet stretching from 1.1 ppm to 1.7 ppm which integrated to 24 hydrogens and accounted for the remaining hydrogens of the compound. The most significant difference between the NMR spectrum of the oxidized, isolated compound and that of α-tocopherolquinone (4) was the doublet in the region of 0.9 ppm. With the isolated compound, this integrated to 15 hydrogens *versus* 12 hydrogens for α-tocopherolquinone. This indicates, in the case of the isolated compound, all of the five aliphatic methyl groups are nearly equivalent. In contrast, with α-tocopherolquinone, only four of the five methyl groups showed this equivalence (4).

All of the data indicated that the isolated endogenous donor was a deoxy derivative of α-tocopherolquinone. The mass spectrum confirmed this structure. No peaks were observed above *m/e* 430. Significant peaks were assigned as follows: *m/e* 359 ($M+ - C_5H_{11}$), the molecular ion minus a terminal dihydroisoprene unit, and *m/e* 289, *m/e* 219, and *m/e* 149, each representative of the loss of an additional dihydroisoprene unit. The signal at *m/e* 149 was the base peak and represents a trimethylbenzoquinone ion. Peaks at *m/e* 121, *m/e* 93, *m/e* 82, and *m/e* 80 were observed. The former two represent the loss of a pair of carbon monoxides from the quinone ring as expected (9). The latter two are those expected

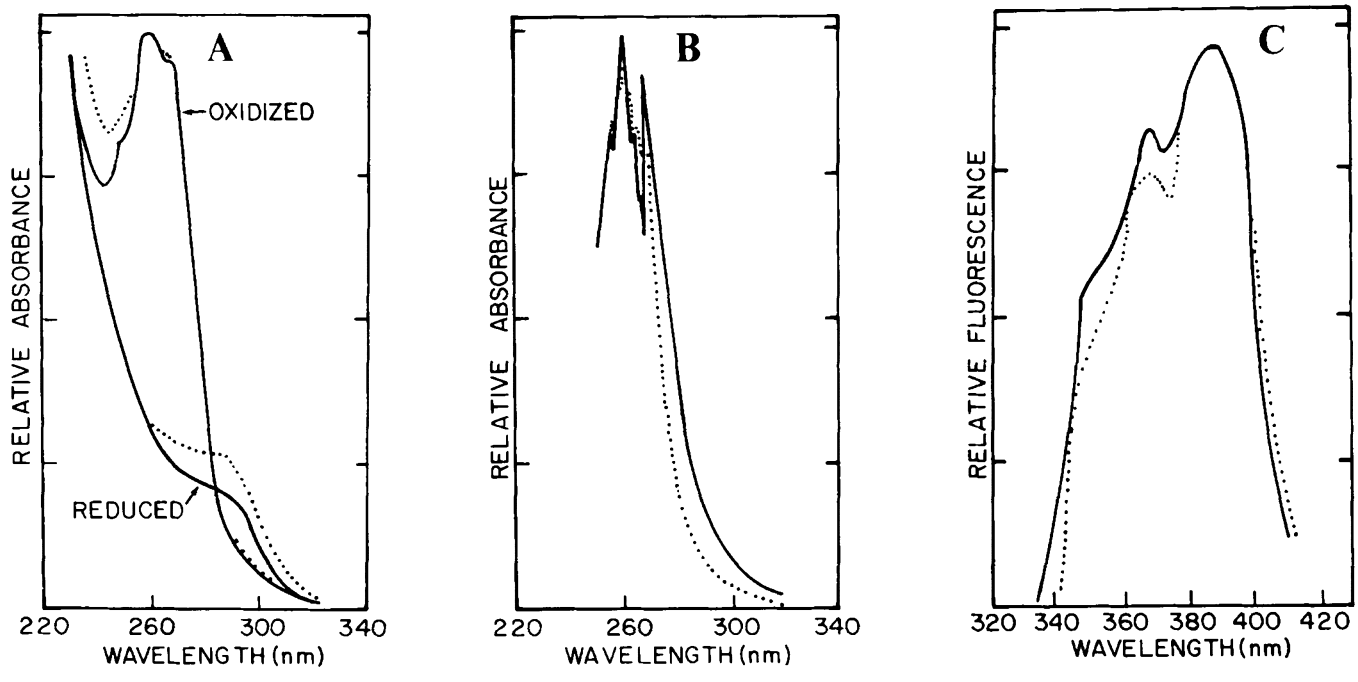

FIG. 1. **Spectrophotometric comparison of isolated and synthetic deoxy-α-tocopherolquinol.** *A*, absorption spectrum of the isolated deoxy-α-tocopherolquinol (——) in absolute ethanol was recorded, and then the compound was oxidized by gentle agitation of the cuvette in air. The absorption spectrum of the oxidized quinol was then obtained. The absorption spectrum of the synthetic deoxy-α-tocopherolquinol (· · · ·) in absolute ethanol was obtained and then similarly oxidized in air and the spectrum recorded. Spectral curves were normalized by computer to the absorbance at 259 nm. *B*, fine structure absorption spectra for the isolated deoxy-α-tocopherolquinol oxidized to deoxy-α-tocopherolquinone by air (——) and the synthetic deoxy-α-tocopherolquinone (· · · ·) in a solution of chloroform/ethanol (2:1). Curves were normalized to absorbance at 262 nm. *C*, fluorescence spectra of isolated deoxy-α-tocopherolquinol oxidized to deoxy-α-tocopherolquinone by air (——) and synthetic deoxy-α-tocopherolquinone (· · · ·) in absolute ethanol. Excitation wavelength was 330 nm. Curves were normalized to the intensity at 389 nm.

from the breakdown of a 1,4-benzoquinone ring (10). All fragments from *m/e* 149 to *m/e* 80 were identical with the fragmentation of α-tocopherolquinone. Thus, the structure of the oxidized form of the endogenous donor appears to be 2-[3, 7, 11, 15-tetramethylhexadecyl]-3, 5, 6-trimethylbenzoquinone or deoxy-α-tocopherolquinone (*III*, Fig. 4). Consequently, by inference the reduced form would be the corresponding benzoquinol.

Confirmation of Structure by Synthesis—A sample of authentic trimethylphytylbenzoquinone was reduced to deoxy-α-tocopherolquinone by catalytic hydrogenation (7). The synthetic deoxy-α-tocopherolquinone had an absorption spectrum essentially identical with the isolated, oxidized, endogenous donor (Fig. 1*A*). Similarly, the fine structure and fluorescence spectra of the synthetic deoxy-α-tocopherolquinone closely resembled that of the isolated compound (Fig. 1, *B* and *C*). In addition, the NMR and the high and low resolution mass spectra were essentially identical with those of the oxidized, endogenous electron donor.

Reduction of the synthesized deoxy-α-tocopherolquinone to deoxy-α-tocopherolquinol with borohydride gave a compound that cochromatographed with the isolated electron donor in both high pressure liquid chromatography and thin layer chromatography. The synthetic deoxy-α-tocopherolquinol gave the same absorption spectrum as the isolated compound (Fig. 1*A*) and was able to serve as an electron donor in the enzyme system without the addition of dithionite or borohydride.

Stoichiometry of Reactions of Deoxy-α-tocopherolquinol—The stoichiometry of the reduction of *cis*-9,*trans*-11-octadecadienoate by deoxy-α-tocopherolquinol was determined in the anaerobic glove bag. Spectrophotometric determination of the conjugated dienoic acid was performed (3). After anaerobic extraction, the measurements of the oxidation of deoxy-α-tocopherolquinol were made spectrophotometrically on eluants from the thin layer plates that had remained in the anaerobic glove bag until the deoxy-α-tocopherolquinol and deoxy-α-tocopherolquinone were separated. If the reaction mixture was extracted with air-saturated chloroform, the ratio of deoxy-α-tocopherolquinone produced to the conjugated diene that disappeared was 2.1 ± 0.1 (n = 9). If the extraction was carried out with anaerobic chloroform, the ratio was 0.9 ± 0.1 (n = 9).

When the reduction of deoxy-α-tocopherolquinone by NADH was carried out in an anaerobic cuvette using small amounts of enzyme protein in the presence of 0.5% gelatin as a stabilizer, we found NADH reduced deoxy-α-tocopherolquinone to deoxy-α-tocopherolquinol with a ratio of 1.1 ± 0.2 (n = 14). Reduction of deoxy-α-tocopherolquinone did not occur if the enzyme protein was heated to 100°C for 5 min.

Conversion of α-Tocopherolquinone to Deoxy-α-tocopherolquinone—Preliminary experiments showed that when [^{14}C]α-tocopherolquinone was incubated with a cell extract of *B. fibrisolvens* in the presence of NADH, [^{14}C]deoxy-α-tocopherolquinone was found. It was identified by absorption spectrum, fluorescence spectrum, and position on the thin layer plate. In order to better define the enzyme(s) involved in this conversion, fractions from a Sepharose 6B column were incubated with [^{14}C]α-tocopherolquinone in the presence and absence of NADH. In the presence of NADH, major and minor peaks of activity were observed, with the major peak coming 40 ml after the void volume and the minor peak corresponding to the region of the soluble fatty acid reductase (3) (Fig. 2). In the absence of NADH, a single peak of activity appeared that eluted 20 ml after the void volume and clearly represented activity of a different enzyme. The presence of two enzymes indicates a two-step reaction with an interme-

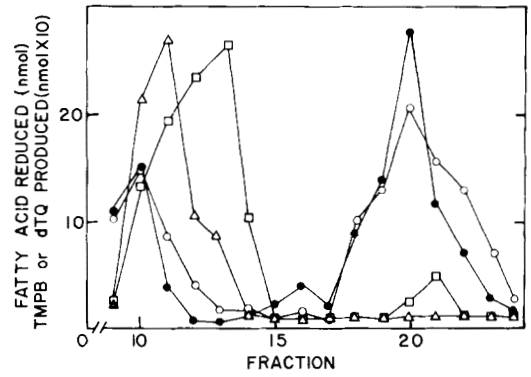

FIG. 2. **Metabolism of α-tocopherolquinone.** Fractions (10 ml) from a Sepharose 6B gel filtration of the cell extract of *B. fibrisolvens* were collected anaerobically. Each fraction (1 ml) was incubated with 10 nmol of [^{14}C]α-tocopherolquinone (68,000 dpm) and with or without 50 μmol of NADH in 0.1 M phosphate buffer, pH 7.4. After 60 min at 37°C, the reaction mixture was extracted with 10 ml of aerobic chloroform/ethanol (2:1) containing a carrier mixture of deoxy-α-tocopherolquinone, α-tocopherolquinone, and trimethylphytylbenzoquinone. The chloroform extract was chromatographed on thin layer plates impregnated with 5% $AgNO_3$ and developed with acetone/toluene/methanol (2:2:1). The fluorescent spots were scraped and the radioactivity of deoxy-α-tocopherolquinone (*dTQ*) (□) and trimethylphytylbenzoquinone (*TMPB*) (△) determined. Similar incubations were carried out for hydrogenation of *cis*-9-,*trans*-11-octadecadienoate using α-tocopherolquinol (●) or deoxy-α-tocopherolquinol (○) as electron donors in the standard assay.

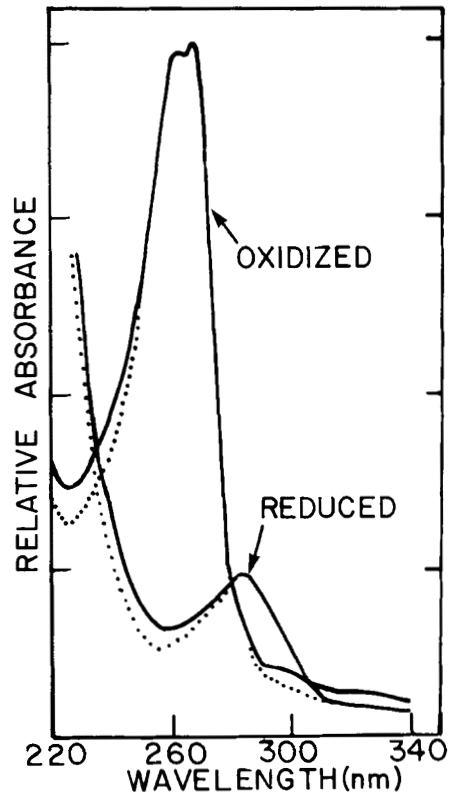

FIG. 3. **Spectrophotometric comparison between trimethylphytylbenzoquinone and the isolated quinone intermediate.** Trimethylphytylbenzoquinone was reduced with borohydride and the intermediate quinone was isolated after incubation with the enzymes from *B. fibrisolvens* without NADH. The absorbance of both the isolated quinone (——) and authentic trimethylphytylbenzoquinone (· · ·) in ethanol was obtained, and each was oxidized to the quinone form by gentle agitation in air. Curves were normalized by computer to the absorbance at 268 nm for each quinone.

diate compound. Considering the changes taking place, it is logical to suspect that the first reaction is a dehydration followed by reduction of a double bond. It follows, therefore, that the suspected intermediate could be trimethylphytylbenzoquinone or its isomer.

Characterization of the Intermediate as Trimethylphytylbenzoquinone—To characterize this intermediate, α-tocopherolquinone was incubated with the first three Sepharose 6B column fractions in the absence of NADH. When [^{14}C]α-tocopherolquinone was used, a radioactive substance was detected on the $AgNO_3$-impregnated thin layer plate that had an R_F of 0.55, corresponding to that of trimethylphytylbenzoquinone. The absorption spectrum of the intermediate eluted from the thin layer plate with ethanol exhibited absorption maxima at 262 nm and 268 nm, and it was essentially identical with the spectrum of authentic trimethylphytylbenzoquinone (Fig. 3). The presence of a double bond was confirmed by the mass spectrum of the intermediate after catalytic deuteration, showing a molecular ion at *m/e* 432.

Dehydration of α-tocopherolquinone could be expected to produce a compound with the double bond in either the 2',3' position or the 3',4' position (Fig. 4). The choice between these two possibilities could be made from the mass spectrum fragmentation pattern of the deuterated compound. This pattern was identical with that obtained on deuteration of trimethylphytylbenzoquinone, and it clearly shows the presence of 1 deuterium atom at the 2' position and the 2nd deuterium atom at the 3' position (Table I).

Final confirmation of the identity of the intermediate as trimethylphytylbenzoquinone was provided by the finding that either authentic trimethylphytylbenzoquinone or the ^{14}C-labeled intermediate formed deoxy-α-tocopherolquinone when incubated with Fractions 4 and 5 from the Sepharose 6B column (Fig. 2) in the presence of NADH. In each case, the identity of the deoxy-α-tocopherolquinone was authenticated by migration on the $AgNO_3$-impregnated thin layer plates and by its absorption spectrum.

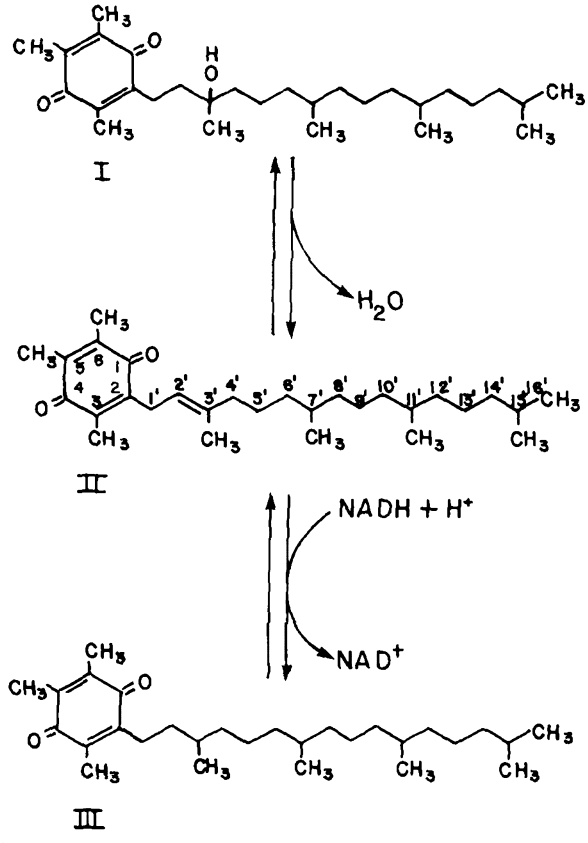

FIG. 4. **Pathway for the conversion of α-tocopherolquinone to deoxy-α-tocopherolquinone.**

TABLE I

Position of deuterium in deuterated trimethylphytylbenzoquinone and the dehydration product of α-tocopherolquinone

Authentic trimethylphytylbenzoquinone (0.5 mg) and the product from the reaction of α-tocopherolquinone with the first three fractions from the gel filtration of the cell extract were simultaneously reduced by deuterium gas in the presence of a platinum catalyst. After isolation, the mass spectral analysis of each was carried out as well as with deoxy-α-tocopherolquinone. The mass distribution for fragments corresponding to the trimethylbenzoquinone ring with successive isoprene side chain carbons is given as percentage distribution of the ion *m/e*, ion *m/e* + 1, and ion *m/e* + 2.

Isoprene chain No.	*m/e*	Deoxy-α-tocopherolquinol	Trimethylphytylbenzoquinone	Intermediate
		%	%	%
None	149	91	88	90
	150	7	9	8
	151	2	3	2
1'	163	94	93	91
	164	4	6	7
	165	2	1	2
1',2'	177	89	32	41
	178	9	60	55
	179	2	8	4
1',2',3'	205	88	31	35
	206	9	11	5
	207	3	58	60
1',2',3',4'	219	93	30	32
	220	6	9	10
	221	1	61	58

DISCUSSION

Thin layer chromatography of a chloroform extract of *B. fibrisolvens* had shown four fluorescent spots (R_F values of 0.03, 0.57, 0.63, and 0.85) capable of reducing *cis*-9,*trans*-11-octadecadienoic acid in the presence of dithionite or borohydride (4). A fifth active compound appeared when the spot at R_F 0.85 was resolved into two compounds by pentane (4). The compound migrating with an R_F of 0.57 had been identified as α-tocopherolquinol, and the substance at R_F 0.85 that did not migrate with pentane had been identified as α-tocopherolquinone (4). Two others have now been identified as deoxy-α-tocopherolquinol and deoxy-α-tocopherolquinone by absorption spectrum, fluorescence spectrum, NMR, and mass spectrometry. The spot with an R_F of 0.63 in the thin layer chromatographic system was identified as deoxy-α-tocopherolquinol, and the spot at R_F 0.85 that migrates with pentane was identified as deoxy-α-tocopherolquinone. From the amount of deoxy-α-tocopherolquinol recovered from the *B. fibrisolvens* cells, the minimal concentration would be 0.09 μM or about one-half the minimal concentration of α-tocopherolquinol (4).

Both α-tocopherolquinol and deoxy-α-tocopherolquinol behave identically in the reduction of *cis*-9,*trans*-11-octadecadienoate. The two hydroquinones show the same activity in the enzymatic reaction. Both show a molecular ratio of two quinones produced per fatty acid reduced when the analysis employed aerobic extraction and a stoichiometry of 1:1 when anaerobic extraction was used. Similarly, identical activity and stoichiometry were shown in the reduction of α-tocopherolquinone and deoxy-α-tocopherolquinone by NADH. It would appear, therefore, that α-tocopherolquinol and deoxy-α-tocopherolquinol occupy the same position in the biohydro-

genation pathway. The difference in stoichiometry between aerobic and anaerobic extraction and the absence of alternate electron acceptors in the hydrogenation system had led to the suggestion that the biohydrogenation pathway involved the oxidation of two α-tocopherolquinols to 2 molecules of the semiquinone (4). These molecules were restored to the reduced state by NADH coupled through a flavoprotein. The results with the deoxy-α-tocopherolquinol reinforce this hypothesis; however, final confirmation rests on detecting the presence of the free radical semiquinones by ESR. This has not yet been achieved.

The formation of deoxy-α-tocopherolquinone from α-tocopherolquinone occurs via the two-step reaction sequence expected for such a transformation (Fig. 4). The first step is the dehydration of α-tocopherolquinone (I) to form trimethylphytylbenzoquinone (II). The second is reduction of the double bond yielding deoxy-α-tocopherolquinone (III), with NADH serving as the reductant. The hydrogenation of an aliphatic olefin without the presence of a neighboring oxygen function is an unusual biochemical reaction. One example of such a system is the biohydrogenation of unsaturated fatty acids, a reaction confined to a few anaerobic bacteria such as *B. fibrisolvens*. A second example is the more widespread hydrogenation of an isoprene double bond, such as occurs in the synthesis of cholesterol and α-tocopherolquinone (5). Although *B. fibrisolvens* is an organism capable of carrying out both hydrogenation reactions, gel filtration of the cell extract indicates that they are catalyzed by different enzymes. Moreover, although NADH served as a primary reductant for both hydrogenation reactions, neither α-tocopherolquinol nor deoxy-α-tocopherolquinol, electron donors for the biohydrogenation of unsaturated fatty acids, could serve as electron donors for the hydrogenation of the isoprenoid double bond.

The discovery of deoxy-α-tocopherolquinone and deoxy-α-tocopherolquinol in extracts of *B. fibrisolvens* appears to be the first account of their occurrence in living organisms. Similarly, the intermediate, trimethylphytylbenzoquinone, has not been previously observed in living organisms, although the closely related compound dimethylphytylbenzoquinone (phytylplastoquinone) has been isolated from solvent extracts of *Euglena gracilis* (11). We have not yet attempted to investigate the occurrence of deoxy-α-tocopherolquinone or deoxy-α-tocopherolquinol in other organisms; however, thin layer chromatography of extracts of animal tissues failed to show a fluorescent spot corresponding to deoxy-α-tocopherolquinone (5). Consequently, the deoxy compounds are not as widely occurring as α-tocopherolquinone and α-tocopherolquinol, which were present in extracts of rat tissues (5).

Thus far, the biohydrogenation of *cis*-9,*trans*-11-octadecadienoate is the only enzymatic oxidation-reduction reaction established for either α-tocopherolquinol or deoxy-α-tocopherolquinol. This is an unusual reaction confined to a few anaerobic bacteria. The wide distribution of α-tocopherolquinol suggests that it probably plays a role in other oxidation-reduction systems. This notion is substantiated by the observation that α-tocopherolquinone is actively reduced by liver mitochondria in the presence of NADH or NADH-generating substrates (5). Since α-tocopherolquinol, deoxy-α-tocopherolquinol, and a pathway for their interconversion are found in *B. fibrisolvens*, it would seem likely that the two quinones are involved in different metabolic reactions in this organism.

Acknowledgment—We wish to acknowledge the expertise and assistance of Fred Williams of the Research Triangle Institute in the mass spectrometry phases of the research.

REFERENCES

1. Kepler, C. R., Hirons, K. P., McNeill, J. J., and Tove, S. B. (1966) *J. Biol. Chem.* **241,** 1350–1354
2. Rosenfeld, I. S., and Tove, S. B. (1971) *J. Biol. Chem.* **246,** 5025–5030
3. Hunter, W. J., Baker, F. C., Rosenfeld, I. S., Keyser, J. B., and Tove, S. B. (1976) *J. Biol. Chem.* **251,** 2241–2247
4. Hughes, P. E., and Tove, S. B. (1980) *J. Biol. Chem.* **255,** 4447–4452
5. Hughes, P. E., and Tove, S. B. (1980) *J. Biol. Chem.* **255,** 7095–7097
6. Morris, S. G., Magidman, P., and Herb, S. F. (1972) *J. Am. Oil Chem. Soc.* **49,** 505–507
7. Lees, M. B. (1957) *Methods Enzymol.* **3,** 355
8. Barr, R., and Crane, F. L. (1963) *Methods Enzymol.* **23C,** 372–408
9. Thompson, R. H. (1971) *Naturally Occurring Quinones*, pp. 77–89, Academic Press, New York
10. Thompson, R. H. (1971) *Naturally Occurring Quinones*, p. 64, Academic Press, New York
11. Whistance, G. R., and Threlfall, D. R. (1970) *Phytochemistry* **9,** 213–219