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Identification of an Endogenous Electron Donor for Biohydrogenation As α-Tocopherolquinol*

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Four fluorescent compounds present in solvent extracts of *Butyrivibrio fibrisolvens* could serve as electron donors for the biohydrogenation of *cis*-9,*trans*-11-octadecadienoate in the presence of dithionite, which was itself inactive. One of the compounds was identified as α-tocopherolquinol and another as α-tocopherolquinone. A partially purified soluble enzyme preparation from *B. fibrisolvens* catalyzed the reduction of α-tocopherolquinone to α-tocopherolquinol in the presence of NADH with a stoichiometry of 1:1. The ratio of α-tocopherolquinone produced to fatty acid reduced was 2:1 when the tocopherol derivatives were extracted aerobically. When the extraction was carried out anaerobically, the ratio was 1. It is suggested that the oxidation of 2 molecules of α-tocopherolquinol, each to the semiquinone, provides the electrons required for the reduction of the *cis*-bond of the conjugated dienoic fatty acid. Although α-tocopherol, phylloquinol, and reduced menadione are inactive, ubiquinol-*4*, ubiquinol-*10*, and trimethylhydroquinone show about one-half the activity of α-tocopherolquinol. Plastoquinol and trimethylphytylbenzoquinol are as active as α-tocopherolquinol.

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). While the reducing hydrogens could come from water (2), NADH, reduced methyl viologen, and an uncharacterized endogenous electron donor could each serve as a source of the 2 electrons involved in this reduction (3). A soluble flavoprotein which catalyzed the double bond reduction using the endogenous electron donor could be isolated from the extract by gel filtration on Sepharose 6B (3). We have recently found that extracts of *B. fibrisolvens* contained relatively large amounts of dithionite (4). Dithionite could not directly serve as an electron donor but did supply electrons to the system via the endogenous donor (4). In this paper, we report the isolation of the endogenous electron donor and its identification as TQH_2.[1]

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[1] The abbreviations used are: TQH_2, α-tocopherolquinol; TQ, α-tocopherolquinone; TQH, α-tocopherolsemiquinone; PQ, plastoquinone; Q-*4*, ubiquinone-*4*; Q-*10*, ubiquinone-*10*; K, phylloquinone; T, α-tocopherol.

EXPERIMENTAL PROCEDURES

Materials

Methyl viologen dichloride, sodium dithionite, sodium borohydride, T, K, and menadione were purchased from Sigma. Authentic TQ, Q-*4*, Q-*10*, and trimethylphytylbenzoquinone were generous gifts of Hoffmann-La Roche. Plastoquinone A was isolated from *Catalpa bignonioides* leaves by the modified method of Bucke and Halladay as described in Ref. 5, and authenticated by spectrophotometric assay (5). Solutions of quinones or hydroquinones, formed by sodium borohydride reduction of the appropriate quinone, were stored in ampules under oxygen-free nitrogen in a light-tight container at −20°C. Trimethylhydroquinone was purchased from Aldrich. All other reagents were purchased from Fisher and, unless stated otherwise, were of analytical grade. Methyl *cis*-9,*trans*-11-octadecadienoate was prepared by reduction of methyl *trans*-11-octadecen-9-ynoate (6). The free acid was obtained by hydrolysis with 2 N KOH under nitrogen.

Methods

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

B. fibrisolvens was cultured and the cell extract was prepared as previously described (4). Two hundred milliliters of the cell extract were placed in a boiling water bath for 10 min and the precipitated material removed by centrifugation at 10,000 × *g* for 20 min. The supernatant was extracted twice with 500-ml portions of chloroform/ethanol (2:1). The pooled organic phases were concentrated to 50 ml in the dark at about 35°C and a portion spotted on a thin layer plate. The thin layer chromatogram was developed by a solvent mixture of acetone/toluene/methanol (2:2:1) in one direction and by *n*-pentane in the other direction. Spots were visualized by fluorescence under light at 366 nm. The concentrated extract was chromatographed with a Waters Associates LC Prep 500 HPLC unit with a C-18 column using 85% methanol (chromatography grade) at a flow rate of 100 ml/min. Bands identified by change in the index of refraction were collected and concentrated, and preliminary identification was achieved by thin layer chromatography as described above. In addition, 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 combined fractions of the second peak of activity from a Sepharose 6B column (3). The enzyme solution was stored at −20°C and prior to use was passed through an anaerobic column (1 × 10 cm) of Sephadex G-25 with 0.1 M phosphate, pH 7.0, as the eluant. The void volume fraction was collected and concentrated. The assay mixture consisted of 70 μg of enzyme protein in 0.5 ml of 0.1 M phosphate, pH 7.0, containing 50 nmol of potassium *cis*-9,*trans*-11-octadecadienoate and variable amounts of an electron donor in 10 μl of ethanol. Sometimes 50 μmol of either sodium dithionite or sodium borohydride was added to the reaction mixture. Incubation was usually for 20 min at 37°C, after which the fatty acids were extracted and the unreacted, conjugated dienoic acid was determined spectrophotometrically (3).

Elemental analysis was performed by Integral Microanalytical Laboratories Inc., Raleigh, NC. High and low resolution mass spec-

trometry was carried out by the Research Triangle Institute, Research Triangle Park, NC. Infrared spectra were recorded on either a Beckman IR-8 or a Perkin-Elmer model 521 spectrophotometer. The NMR spectrometry was carried out by a Varian HA-100 spectrometer in deuterated chloroform containing tetramethylsilane as an internal standard. Samples were washed with D_2O prior to recording the NMR spectrum. Fluorescence was recorded on a SLM fluorometer and absorption spectra were obtained on a Cary model 15 spectrophotometer. Analysis of fatty acids was carried out on the methyl esters, prepared with diazomethane, by gas-liquid chromatography on a 6-foot column of 10% diethylene glycol succinate (Supelco) at 180°C with a Hewlett-Packard computerized 5720A gas chromatograph.

The method of Dilley and Crane (7) was used to synthesize TQ from T. Reduction of the TQ to TQH_2 was carried out by treatment with 0.25 M sodium borohydride in ethanol. The TQH_2 was extracted with chloroform and excess borohydride removed by washing with water. The washed chloroform extract was then subjected to liquid chromatography as described. The TQH_2 was oxidized to TQ by gentle agitation with air.

The TQH_2 isolated from the cell extracts of *B. fibrisolvens* was converted to T by the reductive cyclization method of Tishler and Windler (8). The reaction mixture was extracted with petroleum ether, dried over anhydrous Na_2SO_4, and concentrated by a stream of oxygen-free nitrogen. The reaction products were separated by thin layer chromatography.

The eluant showed the characteristic absorption spectrum of T (9). The ethanolic solution was then reconverted to TQH_2 by successive treatment with $HAuCl_3$ (7) and reduction with borohydride. The presence of TQH_2 was verified by its absorption spectrum and activity as an electron donor in the enzymatic assay. Protein was measured by the method of Lowry as described in Ref. 10. The growth medium was freed of T or T derivatives by extraction four times each with 0.67 volume of chloroform. Residual chloroform was then removed from the growth medium by gassing with oxygen-free nitrogen at 40°C for 2 h. Volatile fatty acids of the medium were restored to the medium before use.

Escherichia coli cells (ATCC 9637) were grown either aerobically or anaerobically for 30 h at 37°C on the minimal salts medium of Vogel and Bonner (11). They were harvested and the cell extract was prepared, extracted, and chromatographed on thin layer plates in exactly the same manner as was done with *B. fibrisolvens*. The criteria for identification of TQH_2 and TQ were fluorescence spectrum, UV spectrum, and electron donor activity in the standard assay. In addition, *E. coli* cells grown in growth medium for *B. fibrisolvens* were treated in the same way.

Determination of Stoichiometry—In the first experiment, the stoichiometry of the reduction of *cis*-9,*trans*-11-octadecadienoate by TQH_2 was determined by incubation of the standard assay system with amounts of TQH_2 ranging from 15 nmol to 84 nmol, but with incubation time increased to 60 min. The 0.5-ml reaction mixture was extracted with 2 ml of a mixture of isopropanol, isooctane, and 1 N H_2SO_4 (40:10:1). The phases were separated by the addition of 1 ml of isooctane and 1 ml of water and the absorption spectrum of the isooctane phase was recorded. From the absorbance at 262 nm, 281 nm, and 233 nm, the amounts of TQ, TQH_2, and conjugated dienoic fatty acid were calculated using extinction coefficients obtained from the absorption spectra of appropriate standards. In a second study, the volume of the incubation mixture was increased to 3 ml, with the amount of enzyme and *cis*-9,*trans*-11-octadecadienoate increased 6-fold. The amount of TQH_2 ranged from 100 nmol to 440 nmol. After 60 min, 1 ml was removed and then extracted as described, but with double the isopropyl alcohol, isooctane, and 1 N H_2SO_4 mixture. For phase separation, 2 ml of water and 2 ml of isooctane containing 100 μg of pentadecanoic acid, as an internal standard, were added. Fatty acids were then analyzed by gas-liquid chromatography. An additional 2 ml of the incubation mixture was extracted with chloroform and the TQ was determined by the method of Dilley and Crane (7). In a third experiment, the conditions of the first experiment were repeated, except that the reaction mixture was continuously gassed with oxygen-free nitrogen and extracted with 1 ml of deoxygenated chloroform. The chloroform extract was transferred to a pregassed cuvette equipped with a serum stopper by means of a gas-tight syringe, and the amounts of conjugated fatty acid and TQ were determined spectrophotometrically.

For the stoichiometry of the reduction of TQ by NADH, the reaction mixture contained 30 μg of enzyme protein, 10 mg of gelatin, 273 nmol of NADH, and 3 μl of ethanol containing 26 nmol to 270 nmol of TQ in a final volume of 2 ml of 0.1 M phosphate, pH 7.4. Oxidation of NADH was measured by change in absorbance at 340 nm, and the TQ reduced was measured spectrophotometrically at 262 nm on an anaerobic chloroform extract of the reaction mixture.

RESULTS

Isolation of Endogenous Electron Donor(s)—It was previously observed that the endogenous electron donor for the *cis*-9,*trans*-11-octadecadienoate reductase exhibited a blue fluorescence (12), which suggested that this compound(s) might be a hydroquinone. Since most of these compounds are lipophilic, a chloroform/ethanol extract of the cell extract was tested and found to possess electron donor activity in the presence of sodium borohydride or sodium dithionite. When the extracted material was concentrated and chromatographed on thin layer silica gel plates, six fluorescent spots were observed (Fig. 1). Each of these was scraped, eluted with chloroform/ethanol, and assayed for activity in the presence of sodium dithionite. Previous studies had shown that dithionite, although inactive by itself, could reduce the oxidized form of the endogenous electron donor (4). Hence, the dithionite was added to the assay mixture to ensure detection of the electron donor even if in an oxidized form. Four of the six fluorescent spots had significant activity (Fig. 1). Two of these, showing a blue fluorescence, had R_F values of 0.63 and 0.85. The two other active spots fluoresced yellow and had R_F values of 0.03 and 0.57. If the thin layer plate, after development with the mixed solvent system (acetone, toluene, and methanol), was developed in a second direction with pentane, the fluorescent spot closest to the front separated into two fluorescent spots, only one of which migrated with pentane.

One of these fluorescent compounds was isolated in milligram amounts by preparative liquid chromatography. The refractive index detector pattern showed an isolated, single symmetrical peak emerging at 1.6 void volumes. Although

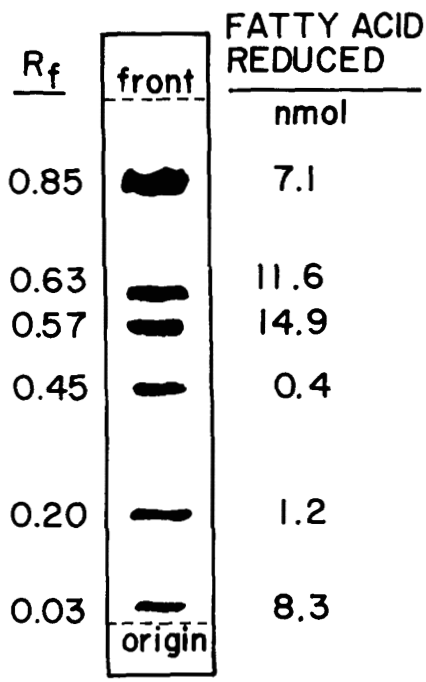


FIG. 1. **Electron donation activity of fluorescent compounds in the cell-free extract.** A chloroform:ethanol extract of a cell extract of *B. fibrisolvens* was concentrated anaerobically and spotted on thin layer plates of silica gel (0.5 mm thick). Development in acetone:toluene:methanol (2:2:1) gave six fluorescent spots when viewed under 366 nm light. The spots were scraped, eluted with ethanol, and then assayed for electron donating activity in the presence of dithionite.

sodium borohydride or dithionite was usually included in the assay reaction mixture, the isolated substance was active without their addition. When subjected to thin layer chromatography, it gave a single blue fluorescent spot with an R_F of 0.63. No other spots appeared on charring the thin layer plate with H_2SO_4. Thus, the isolated compound was presumed to be pure and in the reduced form.

Characterization of the Endogenous Electron Donor—Elemental analysis showed the absence of sulfur, nitrogen, and phosphorus. High resolution mass spectrometry showed it to have the empirical formula of

$C_{29}H_{50}O_3$

Nuclidic mass calculated: 446.3759
Found: 446.3770

The absorption spectrum in absolute ethanol showed a peak at 281 nm with a minimum at 262 nm (Fig. 2*A*). When shaken with air, the spectrum changed, giving a bicuspid peak with maxima at 262 nm and 268 nm and a minimum at 238 nm (Fig. 2*A*). In a chloroform/ethanol solvent mixture, the oxidized compound gave a spectrum showing fine structure with peaks at 255 nm, 262 nm, and 268 nm (Fig. 2*B*). The fluorescence spectrum of the air-oxidized compound when excited at 326 nm had a peak at 396 nm and a shoulder at 372 nm (Fig. 2*C*).

The reduced compound gave IR absorption maxima at 3650 cm^{-1} and 2960 cm^{-1}, indicative of hydroxyl and aliphatic hydrocarbon moieties, respectively. When oxidized, two additional peaks were observed, one at 1641 cm^{-1} and one at 2890 cm^{-1}, indicative of quinone carbonyl functions (13). Proton NMR spectra of the oxidized form of the compound gave no signal in the region of 6.5 to 7 ppm, indicating the absence of hydrogens on the quinone ring. The signals seen were: (*a*) two signals at 0.87 ppm and 0.93 ppm which integrated for 12 hydrogens and were consistent with the presence of four methyl groups bonded to an aliphatic chain (two of these would be associated with the chain and two with an isopropyl end); (*b*) two signals at 2.10 ppm and 2.15 ppm which integrated for nine hydrogens, consistent with three methyl groups attached to a quinone ring; and (*c*) a triplet ($J = 4$ Hz) at 2.6 ppm that integrated for two hydrogens and was consistent with a methylene carbon attached to a quinone ring. The remainder of the spectrum was associated with a broad multiplet, stretching from 1.1 ppm to 1.9 ppm. This integrated to 25 hydrogens and accounts, within experimental error, for the remaining hydrogens of the compound.

The absorption spectrum of the oxidized form of the compound resembled that of a benzoquinone (14). The NMR data showed that such a quinone ring would be totally substituted, first, by the absence of a ring hydrogen and, second, by revealing four groups bonded to the four noncarbonyl carbons of the quinone ring, *i.e.* the three methyl groups and the methylene group of an aliphatic chain. Therefore, the hydroxyl group indicated by the IR spectrum and accounting for the third oxygen of the empirical formula must be associated with the aliphatic chain. The fact that this chain contains 20 carbon atoms (C_{29} minus the trimethylated benzoquinone ring) and is branched suggested that it might be a substituted phytyl chain. Although a phytyl chain should have five methyl groups, the NMR spectrum showed only four. This could be explained if the hydroxyl group were bonded to a carbon carrying one of the methyl branches, thereby introducing a chemical shift from 0.9 ppm to the multiplet region.

Thus, all of the data indicate the isolated endogenous donor to be a trimethyl hydrophytyl benzohydroquinone with a hydroxyl function bonded to the chain, probably at a carbon atom containing a methyl branch. Overall, the fragmentation pattern of the mass spectrum of the oxidized form of the electron donor confirmed this structure. No peaks were observed beyond *m/e* 446. Significant peaks could be assigned as follows: *m/e* 375 ($M^+ - C_5H_{11}$), the parent ion minus a terminal dihydroisoprene unit; *m/e* 305 ($M^+ - C_{10}H_{21}$), the parent ion minus 2 dihydroisoprene units; *m/e* 235 ($M^+ - C_{15}H_{31}$), the parent ion minus 3 dihydroisoprene units; and *m/e* 221, equivalent to $C_{13}H_{17}O_3$ which was the base peak. A peak at *m/e* 149 ($M^+ - C_{20}H_{41}O$) represented the loss of 4 dihydroisoprene units with the 4th containing an oxygen atom. Finally, peaks were observed at *m/e* 121 and *m/e* 93, representing the loss of a pair of carbon monoxides from the trimethyl quinone as expected (15). Fragments observed at *m/e* 80 and 82 are those expected from the breakdown of a 1,4-benzoquinone ring (15), whose presence was also indicated by the UV spectrum (Fig. 2*A*) (13). Thus, the above data suggested the structure of the endogenous reduced electron donor to be 2-[3-hydroxy-3,7,11,15-tetramethylhexadecyl]-3,5,6-tri-

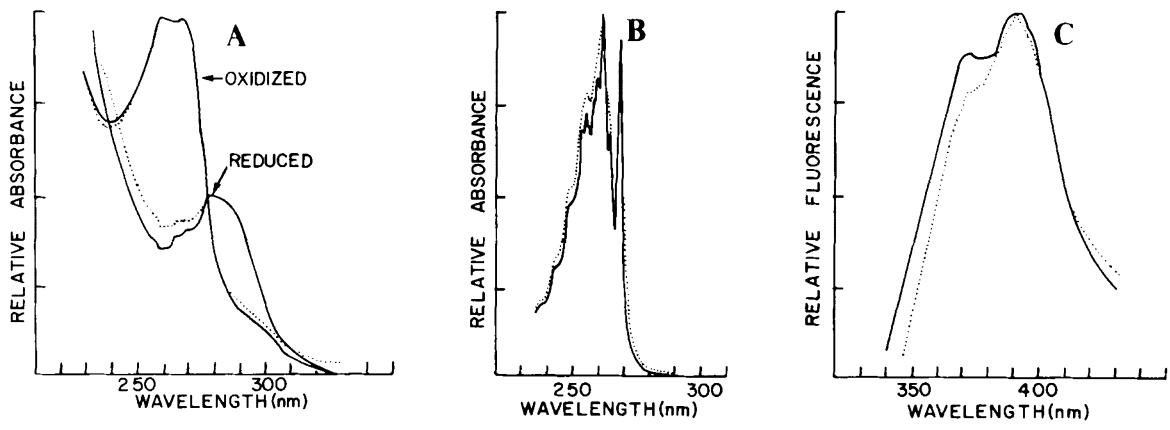


FIG. 2. **Spectrophotometric comparison of isolated and synthetic TQH_2.** *A*, absorption spectrum of the isolated TQH_2 (——) in absolute ethanol was recorded and then the compound was oxidized by gentle agitation of the cuvette in air. The absorption spectrum of the oxidized quinol was then obtained. The absorption spectrum of the synthetic TQ (· · · ·) in absolute ethanol was obtained and then a crystal of sodium borohydride was added and the spectrum again was recorded after 30 s. Spectral curves were normalized by computer to the absorbance at 262 nm for each quinone. *B*, fine structure absorption spectra for the isolated TQH_2 oxidized by air (——) and the synthetic TQ (· · · ·) in a solution of chloroform:ethanol (2:1). Curves were normalized as described above. *C*, fluorescence spectra of isolated TQ oxidized by air (——) and synthetic TQ (· · · ·) in absolute ethanol. Excitation wavelength was 326 nm. Curves were normalized to intensity at 392 nm.

methyl benzoquinol or TQH_2.

Confirmation of Structure by Synthesis—A sample of T was quantitatively oxidized to the TQ by chloroauric acid (7) and then isolated by chloroform extraction and thin layer chromatography ($R_F = 0.85$). It gave an absorption spectrum in ethanol identical with the oxidized form of the endogenous donor (Fig. 2*A*) and in agreement with published spectrum for this compound (7). The fine structure absorption spectrum of the synthetic compound also closely resembled that of the oxidized form of the compound isolated from extracts of *B. fibrisolvens* (Fig. 2*B*), as did the fluorescence emission spectrum (Fig. 2*C*). In addition, the IR, NMR, and mass spectra of the synthetic TQ were essentially identical with those of the oxidized endogenous donor.

Reduction of the synthesized TQ to TQH_2 with borohydride gave a compound that co-chromatographed with the isolated endogenous donor in both liquid chromatography and thin layer systems. Moreover, the absorption spectrum and fluorescence spectrum of the synthetic TQH_2 closely resembled that of the bacterial compound. Both the isolated endogenous electron donor and the synthetic TQH_2 (Table I, lines 1 and 2) could serve as electron donors for the enzymatic reduction of *cis*-9,*trans*-11-octadecadienoate in the absence of sodium borohydride. The synthetic TQ in the absence of borohydride gave 25% of the activity of the synthetic TQH_2, suggesting the presence of reductant for TQ in the enzyme preparation.

Final proof that the isolated endogenous electron donor is TQH_2 comes from the conversion of the isolated compound after air oxidation to T by reductive cyclization (8). The product co-chromatographed on a thin layer plate with authentic T ($R_F = 0.7$). The spot containing the T product was scraped and, when eluted with ethanol, gave an absorption spectrum of T with a maximum at 290 nm (9). The T product was then reconverted to the TQ by chloroauric acid treatment (7) and identified by the expected absorption spectra of the quinone and hydroquinone forms and by activity in the standard enzymatic assay.

Identification of TQ in the Cell Extract—With a solvent system of toluene/acetone/methanol (2:2:1), the synthetic TQ migrated on a thin layer plate to the same R_F as the fastest migrating fluorescent spot of the cell extract. When developed with *n*-pentane, it did not migrate. This suggested that the cell extract contained TQ as well as TQH_2. Further evidence that this was the case was provided as follows. The absorption spectrum of the $R_F = 0.85$ compound from the cell extract that did not migrate with pentane agreed with that of synthetic TQ (Fig. 2*A*) and with the published spectrum (7). Reduction of the compound from the cell extract gave a single spot on thin layer chromatography that coincided with the hydroquinone and gave the hydroquinone absorption spectrum. The isolated substance gave about the same activity (Table I, line 4) as the synthetic TQ in the enzymatic assay in both the absence and presence of borohydride.

Stoichiometry of Reduction of cis-9,trans-11-Octadecadienoate—Initially, two experiments were carried out to determine the stoichiometry of the reductive reaction. In the first, the amount of *cis*-9,*trans*-11-octadecadienoate reduced was determined spectrophotometrically, and in the second, this determination was made by gas-liquid chromatography of the methyl esters. Although in both experiments the TQH_2 oxidized was determined spectrophotometrically on the solvent extract of the reaction mixture, different procedures were used. In the first experiment ($n = 6$), the amount of TQ produced ranged from 2 nmol to 13 nmol. The average ratio of TQH_2 oxidized to *cis*-9,*trans*-11-octadecadienoate reduced was 1.98 ± 0.24. In the second experiment ($n = 9$), the amount of TQ produced ranged from 76 nmol to 125 nmol and the ratio was also 1.98 ± 0.08. In the absence of *cis*-9,*trans*-11-octadecadienoate, only negligible amounts of TQ were produced. The oxidation of 2 TQH_2 molecules yielding 2 electrons over that required for reduction of the double bond could be explained by either of two events. The first is that an endogenous electron acceptor present in the enzyme solution utilized the 2 extra electrons. In order to test this notion, we made use of the enzyme-time relationship. From this relationship, 1 unit of the enzyme incubated for 10 min would be expected to have the same activity as 2 units of the enzyme incubated for 5 min or 5 units of the enzyme incubated for 2 min. If an endogenous oxidant was present in the enzyme solution, the relationship would not hold and more activity would be observed with the higher amounts of enzyme. As can be seen from the results of Table II, the enzyme-time relationship held true, indicating the absence of an endogenous electron acceptor in the enzyme preparation. The second way of accounting for the 2 extra electrons would be that the reduction of the double bond involved the oxidation of 2 TQH_2 to 2 molecules of TQH. The 2 TQH molecules could be oxidized to 2 TQ molecules by atmospheric oxygen during solvent extraction of the reaction mixture. Because of the intense absorption of the enzyme solution, it was not feasible to follow the oxidation of TQH_2 spectrophotometrically without extraction. Using chloroform deoxygenated by prolonged gassing with oxygen-free nitrogen,

TABLE I

Effect of various quinones as electron donors for the hydrogenation of cis-9,trans-11-octadecadienoate

The various compounds were tested in the standard assay system at the indicated concentrations. Incubations were for 20 min at 37°C. The amount of *cis*-9,*trans*-11-octadecadienoate reduced was determined spectrophotometrically, and results are the average of at least two determinations and have been corrected for a blank of 0.8 nmol of dienoic fatty acid lost in the absence of an electron donor.

Addition	Concentration	Fatty acid reduced	
		−$NaBH_4$	+$NaBH_4$
	μM	nmol	
Isolated TQH_2	41	11.1	19.4
Synthetic TQH_2	54	11.3	20.4
Synthetic TQ	58	2.8	20.6
Isolated TQ	52	3.8	17.1
PQ	60		16.5
Trimethylphytylbenzoquinol	50		17.3
Q-4	60		8.6
Q-10	58		10.0
Trimethylhydroquinone	50		8.8
K	48		1.5
Menadione	46		2.1
Hydroquinone	50		3.0
T	36		3.1
FMN	69		3.0

TABLE II

Enzyme-time relationship for the reduction of cis-9,trans-11-octadecadienoate

The indicated amounts of partially purified reductase protein were incubated with 50 nmol each of *cis*-9,*trans*-11-octadecadienoate and synthetic TQH_2 in 0.5 ml of 0.1 M phosphate, pH 7.0, at 37°C. Incubation times were adjusted so that the numerical product of enzyme protein and incubation time was a constant (*i.e.* 810). The amount of dienoic fatty acid reduced was determined spectrophotometrically. Means and standard deviations of triplicate observations are shown.

Protein	Incubation time	Fatty acid reduced
μg	min	nmol
81	10	6.4 ± 0.37
162	5	5.9 ± 1.45
405	2	6.7 ± 0.72

we were able to carry out the extraction anaerobically. With six tubes covering a 10-fold range in the concentration of *cis*-9,*trans*-11-octadecadienoate, we found 0.90 ± 0.15 mol of TQ produced for each mol of fatty acid reduced.

Reduction of TQ by NADH—Previous studies (3) had shown that *cis*-9,*trans*-11-octadecadienoate was reduced by NADH with a stoichiometry of 1:1. Using small amounts of reductase protein in the presence of 0.5% gelatin, we found that NADH reduced the TQ to TQH_2 with a 1:1 stoichiometry (Fig. 3). No reduction occurred if the enzyme solution was heated at 100°C for 5 min.

Specificity of cis-9,trans-11-Octadecadienoate Reductase for Hydroquinones—A series of quinones were tested with the enzyme in the presence of sodium dithionite. PQ and trimethylphytylbenzoquinol were as active at TQ (Table I). Two ubiquinones with *4* and *10* isoprenoid side chains had about one-half the activity of TQ. Trimethylhydroquinone also was one-half as active as TQ, whereas menadione, hydroquinone, T, and FMN were inactive.

Occurrence of TQ—TQ has been reported to occur in a number of mammalian tissues (16). We confirmed these findings by showing its presence (by thin layer chromatography, spectrophotometry, fluorescence, and *cis*-9,*trans*-11-octadecadienoate reductase activity) in adipose tissue, brain, heart, liver, lung, and muscle of rats. Finding TQH_2 or TQ in extracts of *B. fibrisolvens* was surprising since neither T nor its derivatives has been reported in bacteria (17, 18). Accordingly, we examined *E. coli* (ATCC 9637) grown under both aerobic and anaerobic conditions in either a minimal medium or the culture medium for *B. fibrisolvens*. When grown either aerobically or anaerobically in the latter medium, the same four active fluorescent compounds observed in extracts of *B. fibrisolvens* were found in *E. coli* extracts. When grown either aerobically or anaerobically in minimal media, cell extracts chromatographed on thin layer plates showed fluorescent spots coincident with TQ and TQH_2. Since these compounds were found after growth in a glucose-salts medium, it is apparent that *E. coli* can synthesize these compounds. To ascertain whether they were also synthesized by *B. fibrisolvens*, the medium was extracted with chloroform until the solvent-extracted medium showed undetectable levels of T or TQ. Intact cells of *B. fibrisolvens* grown on the extracted medium were able to hydrogenate *cis*-9,*trans*-11-octadecadienoate, and cell extracts had about the same amounts of TQ and TQH_2 as cells grown in the normal medium.

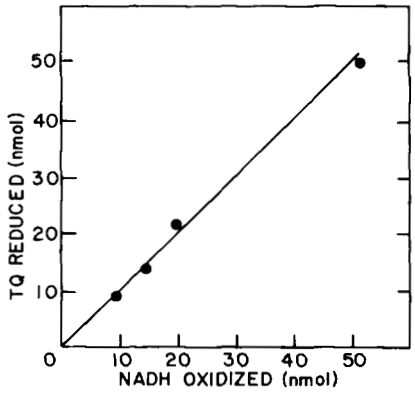


FIG. 3. **Stoichiometry of the NADH reduction of TQ.** The oxidation of NADH was followed spectrophotometrically at 340 nm in a reaction mixture of 30 μg of enzyme protein, 10 mg of gelatin, 273 nmol of NADH, and 0.1 M phosphate, pH 7.4, to a final volume of 2 ml. Amounts of TQ ranging from 26 nmol to 270 nmol were delivered in 3 μl of ethanol. The TQ reduced was determined spectrophotometrically after extraction of the reaction mixture with chloroform under anaerobic conditions.

DISCUSSION

Thin layer chromatography of a chloroform extract of the cell extract of *B. fibrisolvens* showed four fluorescent spots capable of reducing *cis*-9,*trans*-11-octadecadienoate in the presence of sodium borohydride. One of these was isolated and identified as TQH_2 by absorption spectrum, infrared and fluorescence spectra, NMR, and mass spectrometry. In each case, the properties of the isolated compound were the same as the authentic compound. Final proof of identification came from conversion of the oxidized form of the isolated hydroquinone to T. The fastest moving fluorescent spot from the thin layer plate that did not migrate with pentane was similarly identified as TQ. From the amount recovered, TQH_2 was present in the cell extract at a minimal concentration of 0.16 μM.

Although TQ has been shown to occur in animals, plants, shrimp, and limpets (16), we have been unable to find a report of its occurrence in bacteria. Moreover, TQH_2 has been shown to occur in chloroplasts only by indirect means (19). In view of ease of oxidation of the hydroquinone to the quinone and because aerobic extraction procedures had been used, it is possible that the presence of TQH_2 could have been overlooked. Moreover, this problem is confounded by the lability of TQH_2 in light. It is important to stress that in our studies the cell extract of the anaerobe *B. fibrisolvens* contains dithionite (4) and that the extraction was carried out with solvents extensively flushed with oxygen-free nitrogen and under conditions of dim light. Since TQH_2 and TQ were found in extracts of *E. coli* grown on the minimal salts medium and in extracts of *B. fibrisolvens* grown on a medium freed of T derivatives by solvent extraction, it would appear that TQ and TQH_2 were synthesized by the bacteria.

In confirmation of the observations of Green *et al.* (17), we were unable to detect T in extracts of either *E. coli* or *B. fibrisolvens*. This would argue that TQH_2 is formed by hydrogenation of a geranylgeranyl side chain rather than by hydrogenation of α-tocotrienol with subsequent cleavage to TQ and reduction to TQH_2, as has been generally assumed (20, 21).

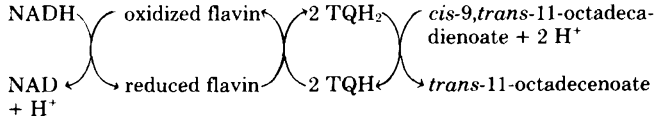
Some appreciation of the specificity of the *cis*-9,*trans*-11-octadecadienoate reductase for the electron donor can be derived from the studies with various quinones added to the reaction mixture with sodium borohydride. Since K, menadione, hydroquinone, FMN, and T were all inactive (Table I), it would appear that a benzoquinone ring is required. Moreover, the inactivity of T indicates that the oxidation-reduction system of T going to TQ or TQH, as once proposed as a possibility for mitochondrial oxidations (9, 22), does not occur in *B. fibrisolvens*. Because the activities of PQ and trimethylphytylbenzoquinol were the same as TQH_2, it can be concluded that the hydroxyl group on the isoprenoid side chain and the methyl group at position 3 are not involved in the association between the electron donor and the enzyme. On the other hand, exchanging two methyl groups for two methoxy groups as in PQ and Q-*4* decreased the activity by one-half. Thus, it would appear that a benzoquinol ring substituted with two methyl groups is the principal aspect of the hydroquinone structure required for enzymatic activity. The isoprenoid side chain is of far less importance. Even it it is completely eliminated, as in the case of trimethylhydroquinone, one-half of the activity remains. Furthermore, no difference in activity is seen between Q-*4* and Q-*10*.

From the experiments carried out thus far, it would appear that the reduction of each *cis*-9,*trans*-11-octadecadienoate involves the oxidation of 2 molecules of TQH_2 to their semiquinone forms. This rests on two observations. Although aerobic extraction of the reaction mixture showed 2 mol of TQ produced for each mole of fatty acid reduced, when the

extraction was carried out anaerobically, the stoichiometry was 1:1. Second, no electron acceptor besides the substrate, *cis*-9,*trans*-11-octadecadienoate, appeared to be present in the reaction mixture. The difference between aerobic and anaerobic extraction could be accounted for by air oxidation of the two TQH to two TQ, whereas with anaerobic extraction, a disproportionation occurs with one TQH reducing the other, yielding TQ and TQH_2. While in the presence of base TQH_2 showed the expected ESR signal, we have not been able to observe an ESR signal in the presence of the enzyme. It is possible that the 2 molecules of TQH are positioned on the enzyme surface so that the spins are paired and, therefore, no ESR signal is obtained.

Although definite proof that TQH is the enzymatic oxidation product must await further experimentation, we tentatively propose two possible pathways for the biohydrogenation of *cis*-9,*trans*-11-octadecadienoate. The formation of *trans*-11-octadecenoate is brought about by 2 electrons from the oxidation of 2 molecules of TQH_2 to 2 molecules of TQH and with the protons of the double bond coming from water (2). In one pathway dithionite could act as primary reductant regenerating 2 molecules of TQH_2 from the 2 molecules of TQH. However, even though dithionite is present in the cells at a minimal concentration of 0.5 mM (4), it would seem more likely that the major role of dithionite is to act as a chemical defense against oxygen for this obligate anaerobe (4).

Previous studies (3) had shown that NADH could act as an electron donor for the reduction of *cis*-9,*trans*-11-octadecadienoate with a stoichiometry of 1:1. We have now shown that the impure, soluble *cis*-9,*trans*-11-octadecadienoate reductase can catalyze the reduction of TQ to TQH_2 with the same stoichiometry. It has also been shown that this same enzyme preparation contains a flavin that is oxidized by *cis*-9,*trans*-11-octadecadienoate and reduced by NADH (3). We, therefore, propose the following pathway for the biohydrogenation of *cis*-9,*trans*-11-octadecadienoate.



The studies reported herein represent the first direct evidence that TQH_2 acts as an electron donor for a biochemical oxidation-reduction system. Although similar systems were proposed more than 20 years ago (9, 23), the primary evidence for this notion lay in the ability of T to reactivate oxidation-reduction reactions in solvent-extracted or aged mitochondria (22, 23). The idea was abandoned when it was shown that the activating effects were nonspecific (24) and more direct evidence that T (or a T derivative) acted as an electron donor did not appear. Several years later, Dilley and Crane (19) showed that levels of TQ and PQ in chloroplasts were altered by light and by addition of NADP and *o*-phenanthroline and suggested that both of these quinones might function as intermediates in photosynthetic electron transport reactions. Whether or not the involvement of TQH_2 as an electron donor occurs widely or is confined to a few reactions in a limited number of organisms is not known. It does seem, however, that our findings suggest that a reinvestigation of such a role for TQH_2 in other systems would be warranted.

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