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Occurrence of α-Tocopherolquinone and α-Tocopherolquinol in Microorganisms†

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Both α-tocopherolquinol and α-tocopherolquinone were found in 56 of 93 strains of microorganisms examined. Organisms that contained these compounds included the single example of a eucaryotic alga, a *Euglena*, and a cyanobacterium (blue-green alga), 22 of 32 genera of bacteria, and 9 genera of yeasts. In the bacteria and yeasts the levels of quinone and hydroquinone were nearly equal and averaged about 3 nmol of each compound g^{-1} of packed cells. Included among the bacteria that contained these compounds were three examples from the newly proposed kingdom of Archaebacteriae. Those microorganisms that did not contain α-tocopherolquinol or α-tocopherolquinone tended to fall into two groups. One group consisted of gram-positive, anaerobic or facultative bacteria with a low content of guanine and cytosine, and the second group encompassed all of the filamentous microorganisms studied. No metabolic function is known for α-tocopherolquinol or its quinone other than as a cofactor in the biohydrogenation of unsaturated fatty acids that can be carried out by only a few organisms.

Until recently, only two quinones were reported to occur in bacteria. These were menaquinones, most frequently associated with gram-positive bacteria, and ubiquinones, generally associated with gram-negative bacteria (1). During the course of investigations on the biohydrogenation of unsaturated fatty acids by the rumen anaerobe *Butyrivibrio fibrisolvens*, we identified two quinol derivatives of α-tocopherol present in the cell extract that could function as electron donors for the reduction of the double bond. One of these was α-tocopherolquinol (6), and the other was deoxy-α-tocopherolquinol (Fig. 1) (8). In addition, the quinone derivatives of both compounds were found (8). Moreover, both α-tocopherolquinol and α-tocopherolquinone (Fig. 1) were present in cell extracts of *Escherichia coli* grown either aerobically or anaerobically (6). Since α-tocopherolquinone and α-tocopherolquinol were found in *Escherichia coli* when grown on a medium containing only glucose and mineral salts and in *Butyrivibrio fibrisolvens* when grown on a medium free of an α-tocopherol derivative, it is clear that each of these organisms could achieve the de novo synthesis of these compounds (6, 8). These findings represented the first report of an α-tocopherol derivative in bacteria although other investigators had sought but failed to detect them (4, 9). This paper presents the results of a more extensive investigation of α-tocopherolquinone and α-tocopherolquinol in bacteria and other microorganisms.

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MATERIALS AND METHODS

Source of microorganisms. Most of the microorganisms were obtained as packed cells, cell suspensions, or mycelial mats from colleagues on the faculty of North Carolina State University and from five other laboratories. The source for each organism is listed in Table 1. The following cultures of rumen bacteria were obtained from M. P. Bryant of the University of Illinois and were grown on the medium we had routinely used for the culture of *Butyrivibrio fibrisolvens* A-38: *Butyrivibrio fibrisolvens* D-1, *Eubacterium ruminantium*, *Lactobacillus vitulinus* GA-1, *Lactobacillus vitulinus* T-185, *Megasphaera elsdenii*, and *Succinivibrio dextrinosolvens*. Cultures of *Peptococcus glycinophilus*, *Peptococcus aerogenes*, and *Clostridium barkeri*, obtained from R. S. Wolfe of the University of Illinois, were grown on a medium that contained 100 ml of water, 0.1 g of NH_4Cl, 0.05 g of KH_2PO_4, 0.05 g of K_2HPO_4, 0.01 g of $MgSO_4 \cdot 7\ H_2O$, 1 ml of Wolfe minerals, and 2 g of yeast extract (Difco). After adjustment to pH 7.2, 0.2 g of $NaHCO_3$ was added, and the solution was boiled and degassed by a stream of oxygen-free N_2:H_2 (95:5) (6), after which 2 ml of 12.5% cysteine-HCl and 12.5% $Na_2S \cdot 9\ H_2O$ were added. In addition, the following substrates, sterilized separately, were added to the medium: for *Clostridium barkeri*, 0.5% glucose; for *Peptococcus glycinophilus*, 0.5% glycine; and for *Peptococcus aerogenes*, 0.5% glucose and 1% monosodium glutamate. Dr. Wolfe

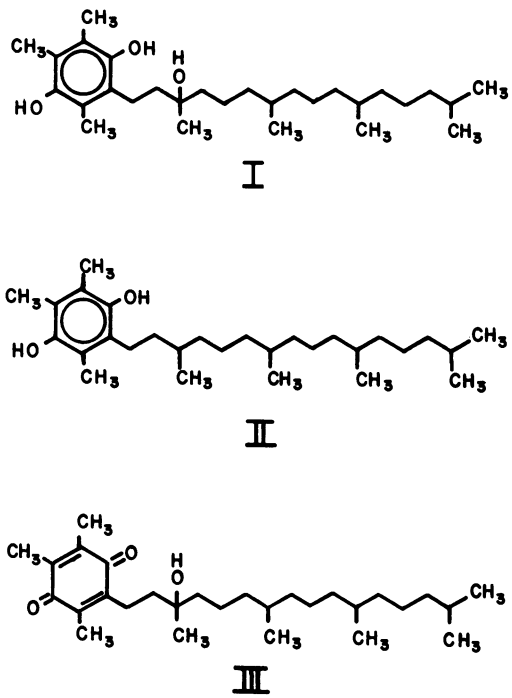

FIG. 1. Structure of (I) α-tocopherolquinol, (II) deoxy-α-tocopherolquinol, and (III) α-tocopherolquinone.

also supplied cultures of *Acetobacterium woodii* and *Sarcina ventriculi* AL-2. The former organism was grown on a similar medium with 0.5% fructose, separately sterilized, as the carbohydrate source and with the yeast extract replaced by a vitamin mixture consisting of 2 μg of biotin, 2 μg of folic acid, 10 μg of pyridoxine, 5 μg of thiamin-HCl, 5 μg of riboflavin, 5 μg of niacin, 5 μg of pantothenate, 5 μg of *p*-aminobenzoic acid, 5 μg of lipoic acid, and 0.1 μg of vitamin B_{12}; $NaHCO_3$ was increased to 0.3 g. The *Sarcina ventriculi* AL-2 was cultured on a medium containing (per 100 ml): 2 g of maltose, 0.2 g of yeast extract, 1.0 g of peptone (Difco), and 0.1 ml of a 0.1% solution of resazurin. The pH was adjusted to 8.5 with cysteine-HCl. The solution was made anaerobic by being boiled and gassed with O_2-free N_2 and autoclaved at 121°C for 20 min. When the solution had cooled, 1 ml of a 5% solution of 0.5% cysteine was added. After being inoculated, the cultures were grown at 37°C for 24 h except for *Sarcina ventriculi* AL-2, which was grown for 12 h. All of these cultures were grown and handled under the strict anaerobic conditions used for *Butyrivibrio fibrisolvens* A-38 (6). The Wolfe mineral solution contained (per liter of solution): 3 g of $MgSO_4$, 0.5 g of $MnSO_4$, 1 g of NaCl, 0.1 g of $FeSO_4$, 0.1 g of $CoCl_2$, 0.1 g of $CaCl_2$, 0.1 g of $ZnSO_4$, 0.01 g of $CuSO_4$, 0.01 g of $AlK(SO_4)_2$, 0.01 g of H_3BO_3, 0.01 g of Na_2MoO_4, and 1.5 g of nitrilotriacetic acid. All of the *Lactobacillus* species except *Lactobacillus vitulinus* GA-1 and *Lactobacillus vitulinus* T-185 were obtained from T. R. Klaenhammer of North Carolina State University and were grown on either Lactobacilli MRS broth (Difco) or APT broth (BBL Microbiology Systems).

TABLE 1. Levels of α-tocopherolquinone and α-tocopherolquinol in various microorganisms

Organism	Source[a]	Level (nmol/g [wet wt]) of: α-Tocopherolquinone	α-Tocopherolquinol	Total
Algae and *Euglena*				
Anabaena cincinalis	G	1.3	0.9	2.2
Euglena gracilis (bleached)	H	5.5	3.5	9.0
Euglena gracilis (green)	H	7.8	5.7	13.5
Selenastrum capricornutum	G	8.6	7.5	16.1
Bacteria				
Acetobacterium woodii	J	0	0	0
Acinetobacter kentucky	A	5.2	6.0	11.2
Alcaligenes faecalis	A	1.7	1.7	3.4
Azotobacter vinelandii	A	3.4	2.9	6.3
Bacillus cereus	A	3.0	2.5	5.5
Bacillus pumilus	A	3.0	3.1	6.1
Bacillus subtilis	A	2.8	1.8	4.6
Butyrivibrio fibrisolvens D-1	I	5.8	5.1	10.9
Butyrivibrio fibrisolvens A-38	N	5.0	3.4	8.4
Caulobacter vibroides	A	2.1	3.8	5.9
Clostridium spp.[b]		0	0	0
Enterobacter aerogenes	A	3.8	3.6	7.4
Escherichia coli	A	3.0	2.4	5.4
Escherichia coli B	A	2.5	3.0	5.5
Escherichia coli B CHS	A	3.3	3.1	6.4
Escherichia coli CSH 36	A	2.7	1.9	4.6
Escherichia coli GI	A	4.0	3.2	7.2
Escherichia coli K-12	A	4.2	1.2	5.4
Escherichia coli Q	A	2.1	1.7	3.8
Eubacterium ruminantium	I	0	0	0
Halobacterium cutirubrum	M	3.0	3.5	6.5
Lactobacillus spp.[c]		0	0	0
Megasphaera elsdenii	I	0	0	0
Methanobacterium bryantii	J	1.9	0.9	2.8
Methanobacterium thermoautotrophicum	J	1.3	0.6	1.9
Micrococcus luteus	A	2.0	1.2	3.2
Micrococcus lysodeikticus	A	3.0	4.2	7.2

TABLE 1—*Continued*

Organism	Source[a]	Level (nmol/g [wet wt]) of: α-Tocopherolquinone	α-Tocopherolquinol	Total
Micrococcus subflavins	A	4.1	5.1	9.2
Mycobacterium smegmatis	A	4.4	2.4	6.8
Mycobacterium vaccae	B	4.1	4.8	8.9
Nocardia asteroides	B	0	0	0
Pediococcus pentosaceus	E	0	0	0
Peptococcus pyrogenes	J	0	0	0
Peptococcus glycinophilus	J	0	0	0
Proteus vulgaris	A	2.8	3.0	5.8
Rhizobium sp. USDA 110-I	F	2.5	3.0	5.5
Rhizobium sp. USDA 110-L	F	4.7	2.0	6.7
Salmonella enteritidis	A	2.5	2.4	4.9
Salmonella newport	A	2.7	3.2	5.9
Salmonella typhimurium LT2	A	4.0	4.7	8.7
Sarcina ventriculi AL-2	J	0	0	0
Serratia marcescens	A	3.0	2.1	5.1
Staphylococcus aureus	A	4.8	2.2	7.0
Staphylococcus epidermidis	A	3.5	3.0	6.5
Streptococcus faecalis	A	2.1	2.8	4.9
Streptococcus faecalis subsp. *liquifaciens*	A	1.8	2.0	3.8
Streptococcus lactis	A	2.2	3.0	5.2
Streptomyces anulatus	B	0	0	0
Succinivibrio dextrinosolvens	I	5.0	3.4	8.4
Thermomicrobium roseum	B	3.7	4.0	7.7
Thermus sp. X-1	B	2.8	3.0	5.8
Treponema sp.	K	3.1	2.4	5.5
Fungi, filamentous[d]		0	0	0
Fungi, yeasts				
Brettanomyces anomalus	E	1.9	2.0	3.9
Candida utilis	E	5.1	4.0	9.1
Cryptococcus albidus	E	2.8	3.0	5.8
Geotrichum candidum	E	2.5	3.7	6.2
Hansenula mrakii	E	3.1	2.0	5.1
Pichia farinosa	E	2.0	3.1	5.1
Saccharomyces cerevisiae	E	4.0	4.1	8.1
Saccharomyces ellipsoideus	E	3.2	2.0	5.2
Saccharomyces fibuligera	E	4.0	1.8	5.8
Schizosaccharomyces pombe	E	4.0	2.8	6.8
Torulopsis etchellsii	E	3.2	3.5	6.7

[a] Sources: A, G. H. Luginbuhl, Dept. of Microbiology; B, J. J. Perry, Dept. of Microbiology; C, P. B. Hamilton, Dept. of Poultry Science; D, T. R. Klaenhammer, Dept. of Food Science; E, H. P. Flemming, Dept. of Food Science; F, G. H. Elkan, Dept. of Microbiology; G, A. M. Witherspoon, Dept. of Botany; and H, J. S. Kahn, Dept. of Biochemistry (North Carolina State University, Raleigh); I, M. P. Bryant, Dept. of Dairy Science; J, R. S. Wolfe, Dept. of Microbiology; and K, C. L. Davis, Dept. of Dairy Science (University of Illinois, Urbana); L, J. Bonaventura, Duke University Marine Laboratory, Beaufort, N.C.; M, M. Kates, Dept. of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada; N, our culture used in previous studies on biohydrogenation.

[b] None of the four *Clostridium* species tested contained either compound (see the text).

[c] None of the seven Lactobacillus strains tested contained either compound when assayed by thin-layer chromatography. However, α-tocopherolquinone was found in *Lactobacillus casei* YIT-0001 (0.7 nmol/g) and in *Lactobacillus plantarum* 14917 (0.4 nmol/g) when high-performance liquid chromatography was used; no α-tocopherolquinol was found in either of these strains.

[d] None of the 17 strains of filamentous fungi examined contained either compound. The strains examined (source) were: *Aspergillus flavus* (C), *Aspergillus ochraceus* (C), *Aspergillus vesicolor* (B), *Asteromyces anulatus* (L), *Bergenerula spartina* (L), *Cunninghamella* sp. (B), *Cunninghamella elegans* (B), *Cunninghamella japonicum* (B), *Fusarium tricintum* (C), *Halosphaeria hemata* (L), *Nia vibrissa* (L), *Nigrospora* sp. (B), *Penicillium citrinum* (C), *Penicillium urticae* (B), *Penicillium zonatum* (B), *Rhizoctonia leguminicola* (C), and *Scopulariopsis brevicaulis* (C).

Analysis of α-tocopherolquinol and α-tocopherolquinone. It is important to emphasize at the outset that, even for cultures grown aerobically, all of the manipulations associated with the analytical procedure were carried out in anaerobic glove bags (I^2R) filled with an N_2:H_2 mixture (95:5) freed of traces of oxygen by passage through copper turnings at 450°C

(6). Similarly, all solutions were freed of oxygen by being boiled and gassed with the oxygen-free N_2:H_2 mixture. All cell suspensions were filtered through a 0.45-μm filter (25 mm; Millipore Corp.) and washed with 10 ml of 0.1 M phosphate, pH 7.0. Packed cells were placed directly on the filter, and mycelial mats were filtered after being blended wth anaerobic phosphate in an Omni mixer (Sorvall). The washed, packed cells were freed of excess fluid by suction, and the weight of the wet cells was determined. The filter and packed cells were added to 10 ml of a mixture of chloroform and ethanol (2:1) and sonicated for 60 s. The chloroform and aqueous phases were separated by the addition of 3 ml of 0.1 M phosphate (pH 7.0) and blending with a Vortex mixer. The chloroform layer was carefully removed and dried by passage through a small funnel containing 1 g of anhydrous Na_2SO_4. The chloroform solution was concentrated by a stream of N_2:H_2 and spotted on a thin-layer chromatoplate coated with 0.5 mm of silica gel H. The thin-layer chromatogram was developed with a solvent mixture of acetone:toluene:methanol (2:2:1). The fluorescent spot indicative of α-tocopherolquinol at R_f 0.6 (6) was scraped and eluted with anhydrous ether. Once the separation was achieved, the plate was removed from the anaerobic glove bag, and the α-tocopherolquinol was allowed to oxidize to its quinone by gentle agitation of the ether solution. The area of the plate from R_f 0.75 to the solvent front was scraped off and eluted with ether. After the ether was removed by a stream of N_2:H_2, the residue was taken up in pentane and chromatographed on a 0.3- by 5-cm column of alumina (Brockman grade III). Elution was accomplished with a stepwise addition of about 10 ml each of 2, 5, 10, 20, and 50% ether in pentane. The fractions eluted by 20 and 50% ether in pentane, containing the α-tocopherolquinone, were dried by the N_2:H_2 stream, and the residue was dissolved in 1 ml of absolute ethanol. The fraction representing the α-tocopherolquinol (i.e., R_f 0.6) was similarly dried and dissolved in absolute ethanol. The amount of α-tocopherolquinone in each fraction was estimated spectrophotometrically at 262 nm by the difference in absorbance before and after the addition of a crystal of $NaBH_4$ (2). The molar extinction coefficient for the absorbance difference was 17,500 M^{-1} cm^{-1}.

In the latter stages of the investigation, a much simpler high-performance liquid chromatography procedure was employed. In this procedure the Millipore filter containing the washed, packed cells was extracted with 10 ml of methanol. After being concentrated, a sample was chromatographed on a Waters C-18 10-μm column, with 97% aqueous methanol used as the eluting solvent. Both α-tocopherolquinol and α-tocopherolquinone were detected by absorbance at 280 nm with a Waters model 441 detector equipped with a Hewlett-Packard model 3380 integrator. In a series of comparisons, both methods gave essentially the same results.

RESULTS AND DISCUSSION

We assayed 93 strains of microorganisms for α-tocopherolquinone and α-tocopherolquinol (Table 1). These comprised one example of each of two genera of algae, one strain of *Euglena gracilis* grown to be photosynthetic and the other lacking chlorophyll, 30 genera of bacteria, 9 genera of yeasts, and 11 genera of filamentous fungi, including 4 of marine origin.

Both α-tocopherolquinone and α-tocopherolquinol were found to be widely distributed among the various microorganisms. In almost every instance we found that the quinone was accompanied by the hydroquinone or vice versa. Moreover, similar levels of α-tocopherolquinone and α-tocopherolquinol were found in all of the miroorganisms. Thus, the average level of α-topcopherolquinone among the 41 strains of bacteria in which it was found was (± standard deviation) 3.2 ± 1.1 nmol g^{-1} of packed cells, and in the yeasts it was 3.3 ± 1.0 nmol g^{-1} of packed cells. The average level of α-tocopherolquinol in the same bacteria was 2.9 ± 1.2 nmol g^{-1} of packed cells, and in the yeasts the α-tocopherolquinol also averaged 2.9 ± 0.9 nmol g^{-1} of packed cells. Slightly higher amounts were found in the two cultures of *Euglena* and in the eucaryotic alga, whereas in the one culture of a cyanobacterium, *Anabaena cincinalis*, the levels of both fell within the range of the other bacteria and yeasts. Thus, the presence and level of α-tocopherolquinone or α-tocopherolquinol could not be correlated with the shape of the organism, Gram stain reaction, or tolerence to oxygen. Since α-tocopherolquinone and α-tocopherolquinol were present in both methanogenic organisms and in the halophile, these compounds are found in organisms that have been classified in the newly proposed kingdom of *Archaebacteriae* based on analysis of their 16S rRNA (3). These findings would indicate that the appearance of α-tocopherol derivatives in procaryotes occurred early in evolution. Moreover, the presence of these benzoquinone compounds in both gram-positive and gram-negative organisms runs counter to the suggestion that naphthoquinones are associated with gram-positive bacteria and benzoquinones with gram-negative bacteria (1).

The wide occurrence of α-tocopherolquinone and its hydroquinone in bacteria raises the question of why it had remained undetected for so long. This question is particularly appropriate, since there have been several investigations on the quinones in bacteria such as those made by Whistance et al. (9). Moreover, Green et al. (4) specifically assayed bacteria for α-tocopherol and its derivatives but failed to detect these compounds. Our initial investigations leading to the isolation and characterization of α-tocopherolquinol as an electron donor for biohydrogenation of unsaturated fatty acids in *Butyrivibrio fibrisolvens* rested on procedures that were gentle, carried out at a neutral pH_2 at relatively low temperatures, under strict anaerobic conditions, and in the absence of bright light (6). We contin-

ued to use these procedures in the studies reported here. These conditions are much less drastic than those employed by other investigators and may account for the difference in the results obtained.

Possibly related to the necessity for gentle extraction procedures is the finding that 52% of the time the levels of α-tocopherolquinone and α-tocopherolquinol were within 80% of each other. The high regularity of their equal occurrence suggests that these values may not have been a reflection of mere cellular concentrations. The same level of each compound could be accounted for also if the majority of α-tocopherolquinone occurred as the highly reactive semiquinone that, under the anaerobic extraction conditions we used, underwent disproportionation, yielding equal amounts of quinone and hydroquinone. Similar equality of the level of quinone and hydroquinone occurred in animal tissues and at equilibrium upon the reduction of α-tocopherolquinone by NADH in mitochondria (7). Thus, it is possible that α-tocopherolsemiquinone may be the principal naturally occurring form of this metabolite.

Neither α-tocopherolquinone nor α-tocopherolquinol was detected in 38 strains of microorganisms that fell into two distinct groups. However, it should be emphasized that the apparent lack of these compounds may simply reflect the methods used and that even in these organisms the α-tocopherol derivatives may exist at levels undetectable by the spectrophotometric method used or may occur in a different form not liberated by simple solvent extraction. This conjecture seems appropriate since, when the more sensitive high-performance liquid chromatography method was used, small amounts of α-tocopherolquinone, 0.4 and 0.7 nmol g^{-1} of packed cells, were found in *Lactobacillus plantarum* 14917 and *Lactobacillus casei* YIT-0001, respectively. No α-tocopherolquinol was detected in either case, but the presence of α-tocopherolquinone was confirmed by mass spectral analysis (6).

For whatever reason, these 38 strains of microorganisms were different from the rest. Among these were 20 strains of bacteria that included *Clostridium barkeri*, *Clostridium pasteurianum*, *Clostridium perfringens*, *Clostridium sporogenes*, *Enterobacter aerogenes*, *Eubacterium ruminantium*, *Lactobacillus brevis*, *Lactobacillus casei* YIT-0001, *Lactobacillus fermentum* 36, *Lactobacillus plantarum* 14431, *Lactobacillus plantarum* 14917, *Lactobacillus vitulinus* GA-1, *Lactobacillus vitulinus* T-185, *Megasphaera elsdenii*, *Nocardia asteroides*, *Pediococcus pentosaceus*, *Peptococcus aerogenes*, *Peptococcus glycinophilus*, *Sarcina ventriculi* AL-2, and *Streptomyces anulatus*. With the exception of *Megasphaera elsdenii*, *Nocardia asteroides*, and *Streptomyces anulatus*, all are gram-positive, anaerobic (or facultative) organisms and have a low percentage of guanine plus cytosine in their DNA, and they all fall into the single group designated *Clostridium* and relatives based on their 16S rRNA sequence (3). On the other hand, there are several organisms listed in this category that did contain α-tocopherolquinone and α-tocopherolquinol. These include *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Streptococcus lactis*.

The second group of microorganisms that apparently lacked α-tocopherolquinone and α-tocopherolquinol are strictly aerobic and are all filamentous organisms. They include the two filamentous actinomycetes examined. *Nocardia asteroides* and *Streptomyces anulatus*, and all seventeen of the filamentous fungi cultures that were assayed. Although the two groups of microorganisms differ in morphology and oxygen requirements, they may have a common ancestral origin, i.e., the gram-positive bacteria, as proposed by Fox et al. (3).

We have shown that α-tocopherolquinone and α-tocopherolquinol were present in *Escherichia coli* grown on a minimal medium and in *Butyrivibrio fibrisolvens* grown on a more complex medium that had been freed of α-tocopherol derivatives by chloroform extraction (6). Therefore, it is apparent that each of these two organisms is capable of synthesizing α-tocopherolquinone and α-tocopherolquinol. Since most of the organisms listed in Table 1 were grown for us by others, it is not known if the media on which they were cultured contained an α-tocopherol derivative. Consequently, despite the fact that a sample of yeast extract and Trypticase did not contain detectable levels of an α-tocopherol derivative, there is no assurance that the medium on which a given organism was grown was similarly devoid of these compounds. Hence, whether the α-tocopherolquinone present in an organism other than the two cited above arose via absorption from the medium or by de novo synthesis remains a question for further study. It should be emphasized that the purpose of this communication has been to report that α-tocopherolquinone and its hydroquinone occur widely in the microbial world.

Thus far, the only known metabolic function for α-tocopherolquinol is its action as an electron donor in the reduction of *cis*-9,*trans*-11-octadecadienoic acid (5, 6), the final reaction in the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens* A-38. Because the biohydrogenation of unsaturated fatty acids is a biochemical process confined to very few rumen bacteria, these quinones must have a different function(s) in the other organisms that contain them. Indeed, in *Butyrivibrio fibrisolvens* the

high cellular level of *cis*-9,*trans*-11-octadecadienoate reductase led to the suggestion (5) that even this enzyme may have a metabolic function other than biohydrogenation of unsaturated fatty acids. Although α-tocopherolquinone was rapidly reduced by NADH in rat liver mitochondria, it did not appear to play a role in the classical electron transport (7). Thus, while it is probable that α-tocopherolquinol (or its quinone) plays a general metabolic role in both procaryotic and eucaryotic organisms, the nature of this role is unknown.

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