

NASA-TM-84382 19830020581

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June 1983

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N83-28852 #

ABSTRACT

The membrane-bound NADH oxidase of Paracoccus halodenitrificans was inhibited by dicoumarol, 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO), and exposure to ultraviolet light (at 366 nm). When the membranes were extracted with n-pentane, NADH oxidase activity was lost. Partial restoration was achieved by adding the ubiquinone fraction extracted from the membranes. Succinate oxidation was not inhibited by dicoumarol or HQNO but was affected by ultraviolet irradiation or n-pentane extraction. However, the addition of the ubiquinone fraction to the n-pentane-extracted membranes did not restore enzyme activity. These observations suggested that reducing equivalents from succinate entered the respiratory chain on the oxygen side of the HQNO-sensitive site and probably did not proceed through a quinone.

In an earlier study (22), exponentially growing cells of the moderately halophilic bacterium Paracoccus halodenitrificans were shown to maintain intracellular concentrations of potassium and sodium (with $K^+ > Na^+$) that were independent of the extracellular concentrations of these ions. Cells taken from the maximum stationary phase were depleted with respect to K^+ and had accumulated Na^+ so that the intracellular concentrations of these ions approximated those found in the growth medium. These changes were also accompanied by changes in the activity of the membrane-bound NADH oxidase, suggesting a correlation between these ion fluxes and the activity of the respiratory chain. In order to gain a better understanding of these phenomena, a study of the electron transport system in P. halodenitrificans was initiated. The patterns of inhibition observed during NADH and succinate oxidation suggested that a menaquinone (MK) was associated with the oxidation of NADH while a ubiquinone (UQ) was involved in succinate oxidation. The presence of MK and UQ in the same organism is unusual and appears restricted to certain enteric bacteria (2,25). Furthermore, the only quinone found in P. halodenitrificans is UQ-9 (4). The results of the experiments described in this paper confirm that UQ-9 is the only detectable quinone in P. halodenitrificans and that NADH and succinate may not be oxidized through the same quinone pool.

MATERIALS AND METHODS

Growth of bacteria and preparation of membranes. P. halodenitrificans (ATCC 13511) was grown aerobically in a complex medium as previously described (22). Stationary-phase cells were harvested by

centrifugation at $4,300\times g$ for 30 min at 4°C and immediately processed. The cells were suspended in 10 mM N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid (HEPES)-500 mM NaCl buffer (pH 7.4) to a cell-buffer ratio of 0.25 (wt/v), passed through a cold French pressure cell operated at 15 kpsi (103 MPa), and the suspension centrifuged at $4,300\times g$ for 30 min at 4°C . The supernatant was centrifuged at $175,000\times g$ for 60 min at 4°C . The red gelatinous pellet was suspended in the HEPES-NaCl buffer and designated as the membrane fraction.

Assays. Oxygen consumption was measured polarographically using a Yellow Springs Model 53 Biological Oxygen Monitor and Model 5331 Oxygen Probe. The oxygen electrode was calibrated as described by Beechey and Ribbons (1) with membranes prepared from *P. halodentrificans* as the source of NADH oxidase. An air-saturated solution of the HEPES-NaCl buffer contained 225 nmol of $\text{O}_2 \text{ ml}^{-1}$ at 30°C .

NADH and succinate oxidase activities were assayed at 30°C in a total volume of 3 ml in the presence of 30 μmol of HEPES (pH 7.4) buffer and 1.5 mmol of NaCl. Depending on which oxidase activity was assayed, either 2.5 μmol of NADH or 15 μmol of succinate were added. The reaction was initiated by the addition of the membrane fraction, either 300 μg or 2 mg of membrane protein, when NADH or succinate oxidase activities were assayed. A unit of oxidase activity was defined as that amount of enzyme catalyzing the consumption of 1 μmol of $\text{O}_2 \text{ min}^{-1}$, after correcting for any endogenous oxygen consumption. The dehydrogenases were assayed at room temperature spectrophotometrically at 410 nm. To determine NADH dehydrogenase activity, the following additions were made in a total volume of 1 ml: 10 μmol HEPES (pH 7.4) buffer, 500 μmol of

NaCl; 150 nmol of NADH; 1 μmol $\text{K}_3\text{Fe}(\text{CN})_6$; and the membrane fraction (approximately 50 μg of membrane protein). After first determining the nonenzymatic rate of ferricyanide reduction, the membrane fraction was added and the initial rate of reduction was determined. Succinate dehydrogenase was assayed in a reaction mixture containing the following additions in a total volume of 1 ml: 60 μmol of potassium phosphate buffer (pH 8.0); 10 μmol of sodium succinate; 1 μmol of $\text{K}_3\text{Fe}(\text{CN})_6$; and the membrane fraction (about 250 μg of membrane protein). A unit of dehydrogenase activity is that amount of enzyme catalyzing the reduction of 1 μmol of ferricyanide min^{-1} , using $1.0 \text{ mM}^{-1} \text{ cm}^{-1}$ for the mM absorption coefficient. All activities were also corrected for any ferricyanide reduction that occurred in the absence of a substrate. In the oxidase and dehydrogenase assays, specific activity was defined as units mg protein^{-1} .

Proteins were determined as described by Lowry et al. (17) using bovine serum albumin (Fraction V) as the standard. Sodium dodecylsulfate, at a final concentration of 0.1%, was added to solubilize the membranes.

Photoinactivation studies. A model 1L SC-1L PenRay Lamp (Ultra-violet Products, Inc.) was used for these studies. With this lamp, 97% of the incident radiation occurred at 366 nm. Appropriately diluted membranes were placed in a tube whose contents were protected from light and irradiated while stirring at 4°C . Aliquots were periodically removed and assayed for the appropriate enzyme activities. Concurrently,

a nonirradiated control was also assayed. No loss of any of the enzyme activities assayed was observed in the control.

Identification of quinones. Quinones were extracted with methanol-acetone (14) or n-pentane (8), and purified by chromatography on acid-washed alumina (24). UQ was determined from the decrease in absorbance at 275 nm following the addition of NaHB_4 using $12.5 \text{ mM}^{-1} \text{ cm}^{-1}$ for the mM absorption coefficient (6).

The number of isoprenoid units was determined by mass spectrometry or reverse-phase thin-layer chromatography. Mass spectra were determined by Frank Church using a DuPont Model 21-491 Mass Spectrometer interfaced with a GCMS computer system, and the chromatographs were determined using 100 μm precoated Avicel microcrystalline cellulose plates from EM Laboratories. The quinones were detected with leucomethylene blue (6). UQ standards were obtained from the Sigma Chemical Company with the exception of UQ-8, which was prepared from Escherichia coli (K-12) membranes in which it occurs as the principle UQ (7).

Incorporation studies. Lyophilized membranes were extracted nine times with n-pentane, using fresh 2-ml portions of the solvent for each extraction. The "depleted" membranes were dried for 1 hr at room temperature in a rotary evaporator and "reconstituted" by addition of the quinone (dissolved in a minimal quantity of n-pentane) to a quinone-to-membrane protein ratio of 300 nmol:mg. The suspension was stirred at 4°C for 30 min, centrifuged at $17,300 \times g$ for 30 min at 4°C, and the supernatant discarded. The pellet was carefully rinsed with n-pentane

and the residual pentane was removed by drying the membranes in a rotary evaporator for 1 hr at room temperature. The quinones used in these experiments were either the UQ standards or the endogenous quinones. The latter were prepared from lyophilized membranes by methanol-acetone extraction (crude quinone) and subsequently purified by acid-alumina chromatography (purified quinone). All operations were carried out in subdued light; fractions containing quinones were protected from light.

RESULTS

Inhibition of oxidase activities. The oxidation of NADH was sensitive to dicoumarol, suggesting that NADH was oxidized through an MK-dependent step; maximum inhibition of oxygen consumption (90%) was observed when the dicoumarol concentration was approximately 50 μM , while 50% inhibition took place in the presence of about 5 μM dicoumarol (Fig. 1a). NADH oxidation was also affected by 2-n-heptyl-4-hydroxy-quinoline-N-oxide (HQNO), an inhibitor of electron transport at the level of cytochrome b (3,13,15,19); maximum inhibition of oxygen consumption (96%) occurred when the HQNO concentration was about 1.5 μM , while 50% inhibition was obtained at approximately 100 nM inhibitor (Fig. 1b). Neither succinate oxidase nor NADH dehydrogenase activities were affected by these inhibitors even when, in the case of succinate oxidase, the concentrations of dicoumarol or HQNO were increased to 300 μM and 12 μM , respectively.

Photoinactivation. The results of an experiment demonstrating the photosensitivity of NADH and succinate oxidases to long-wavelength UV

light are shown in Fig. 2a. NADH oxidase activity disappeared exponentially with a half-life of 18 min, whereas 50% of the succinate oxidase activity disappeared after 40 min. NADH and succinate dehydrogenase activities were also inactivated by 366 nm light (Fig. 2b). The NADH dehydrogenase was inactivated with a half-life of 7.5 hr, whereas succinate dehydrogenase inactivation took place with a half-life of 80 min. Because of the inactivation of succinate dehydrogenase, it was unclear to what extent the photoinactivation of the dehydrogenase contributed to the loss of succinate oxidase activity.

The effect of solvent extraction. Extraction of membranes with either n-pentane or a methanol-acetone removed a UV-absorbing material. The maximum absorption of this material, located at 275 nm, decreased and shifted to 290 nm upon the addition of NaBH_4 , which was suggestive of a UQ. No evidence for the characteristically complex spectrum of an MK (16) was observed in the membrane or in the high-speed supernatant fractions. When MK and UQ occur in the same microorganism, higher concentrations of the former are found in anaerobically grown cells (20,25). Therefore, cells were grown anaerobically in the presence of 1% KNO_3 to increase the levels of MK, if any were present. The membrane and high-speed supernatant fractions from anaerobically grown cells were examined spectrally, but the only material detected resembled that obtained from aerobically grown cells.

Extraction of the membrane fraction with n-pentane resulted in the loss of NADH oxidase activity. NADH oxidase activity in "depleted" membranes was partially restored by adding material extracted with

either methanol-acetone or n-pentane. In addition, UQ-6, UQ-9, or UQ-10 partially restored NADH oxidase activity, each to about the same extent. No reactivation was observed when the vitamin K₁ was added to the depleted membranes. An experiment illustrating reactivation with the endogenous quinone is shown in Table 1. The lyophilized membranes were extracted with n-pentane, which resulted in the loss of about 93% of the NADH oxidase activity and 99% of the quinone (equivalent to removing 9.6 nmol of UQ × mg membrane protein⁻¹). NADH oxidase activity was partially restored by adding either the crude quinone fraction (obtained by methanol-acetone extraction) or the chromatographically purified quinone fraction. Following reactivation, NADH oxidase activity was inhibited by dicoumarol and HQNO (Table 1) to about the same extent as observed prior to depletion (Fig. 1). This indicated that the addition of the quinone fraction restored the respiratory chain rather than bypassing the quinone-depleted site.

Succinate oxidase activity was also affected by n-pentane extraction. However, depletion resulted in only a 28% loss of the initial succinate oxidase activity despite the extraction of 99% of the quinone (Table 1). No reactivation was observed when the crude quinone fraction was restored to the depleted membranes. Solvent extraction also caused a 36% loss of succinate dehydrogenase but none of the NADH dehydrogenase activity. As in the case of the photoinactivation experiments, the inactivation of succinate dehydrogenase could have accounted for the loss of succinate oxidase activity.

Identification of the quinone. When a methanol-acetone extract of membranes was chromatographed on acid-washed alumina, the only quinone detected was eluted with 5% ethyl ether in petroleum ether. MK would have been eluted with 1% ethyl ether in petroleum ether. The absorption spectrum of the quinone was indistinguishable from the spectrum of authentic UQ-10 (6). The maxima before and after NaBH_4 reduction were located at 275 nm and 290 nm, respectively, and the ratio of the two maxima was 2.9.

The length of the isoprenoid chain was identified by reverse-phase chromatography (Fig. 3). The quinone fraction extracted with methanol-acetone (Fig. 3, position 2) and the material purified by alumina chromatography (Fig. 3, position 3) exhibited a single leucomethylene blue-oxidizing spot whose mobility was identical to that observed with authentic UQ-9 (Fig. 3, position 5). The mobility of the quinone obtained from anaerobically grown cells (Fig. 3, position 4) was also similar to UQ-9. The mass spectrum of the quinone exhibited prominent peaks at m/e 235 and 197, which are characteristic features of ubiquinones, and at m/e 794 and 796, which correspond to the parent mass peaks for UQ-9 and its hydroquinone (7,18). No peaks characteristic of UQ-8 and UQ-10 were observed at m/e 726 and 826.

DISCUSSION

Ubiquinones occur in a variety of bacteria where they participate as hydrogen carriers linking the dehydrogenases (e.g., NADH and succinate) to the cytochrome portion of the respiratory chain (11). It is

not clear how ubiquinones are organized in the electron transport chain; there is some question if ubiquinones exist as a mobile and homogeneous pool (9,21).

The results described in this paper were consistent with the notion that NADH was oxidized through UQ-9. Although NADH oxidation was inhibited by dicoumarol, no MK was detected either spectrally or chromatographically. Dicoumarol also inhibits UQ-dependent reactions, such as the NADH-UQ segment of the mitochondrial respiratory chain (10) and the NADH oxidase of Aerobacter aerogenes, where the only quinone present is UQ-8 (12). Therefore, the inhibition of NADH oxidase activity by dicoumarol need not reflect an MK-dependent step in the oxidation of NADH. HQNO inhibits succinate oxidase activity at a site between UQ and oxygen (15). The absence of HQNO inhibition of succinate oxidase activity, and the apparent refractoriness of succinate oxidase activity to photoinactivation and solvent extraction, suggested that succinate was not oxidized through a UQ-dependent step.

If we postulate that UQ did not exist as a mobile pool in P. halodenitrificans, then the differences observed in the oxidation of NADH and succinate can be reconciled with the notion that succinate is oxidized through UQ-9. Cox et al. (5) proposed that in Escherichia coli, UQ associated with non-heme iron, occurred at two sites, bridged by cytochrome b. During NADH oxidation both quinones are reduced, whereas during the oxidation of D-lactate, only the UQ on the oxygen side of the cytochrome is reduced. Such a protein-associated UQ would not behave as if it were part of a pool. Trumpower (23) makes the point

that if UQ were bound to a protein in the respiratory chain, such a quinone should have altered chemical and physical properties. Whether such binding would confer the kinds of differences we have observed is not clear.

At the present time, we cannot explain the behavior of succinate oxidation with respect to HQNO inhibition and quinone depletion. Studies are currently under way to determine if succinate is oxidized through UQ, or if UQ does not exist as a homogeneous and mobile pool.

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TABLE 1. Restoration of oxidase activities following
extraction with n-pentane^a

Fraction	Specific activity			
	Oxidase		Dehydrogenase	
	NADH	Succinate	NADH	Succinate
Lyophilized	0.462	0.017	5.0	0.188
Depleted	0.032	0.012	5.1	0.121
Reconstituted				
Crude quinone	0.295	0.011	-	-
Purified quinone	0.165	-	-	-
Reconstituted, crude	0.267			
quinone		-	-	-
+ Dicoumarol	0.024	-	-	-
+ HQNO	0.027	-	-	-

^aMembranes (containing 4.17 mg of protein) were lyophilized and extracted with n-pentane. The extracted membranes were treated with either crude or purified quinone (equivalent to 1.2 μ mol of UQ) and reactivated as described in the Materials and Methods section. When present, the concentrations of dicoumarol and HQNO were 50 μ M and 1 μ M, respectively.

Figure Captions

FIG. 1. Inhibition of NADH oxidase activity. 100% maximum sp act is equivalent to 0.391 (A) and 0.417 (B). In these experiments 280 μ g of membrane protein was added. When NADH oxidase activity was assayed in the presence of 10 μ l of ethanol (the solvent used to dissolve HQNO and the quantity added at each HQNO concentration), a slight stimulation of NADH oxidase activity was observed. This is reflected in the higher specific activity of the membrane fraction in (B).

FIG. 2. Photoinactivation of NADH and succinate oxidation. Membranes were diluted in 10 mM HEPES-500 mM NaCl buffer (pH 7.4) to give a final protein concentration of 2 mg ml⁻¹, and 7 ml of the membrane suspension were irradiated at 4°C. Aliquots were periodically removed, and assayed for NADH (○) and succinate (●) oxidase activities (A) and dehydrogenase activities (B). The initial specific activities for (A) were 0.239 and 0.033, respectively; those for (B) were 2.6 and 0.15, respectively.

FIG. 3. Reverse phase chromatography of ubiquinones. The following materials were chromatogrammed: (1) a standard mixture containing UQ-6, UQ-7, UQ-8, UQ-9, and UQ-10; (2) the crude quinone fraction, obtained by methanol-acetone extraction; (3) the quinone fraction after purification by acid-alumina chromatography; (4) the crude quinone fraction from anaerobically grown cells; and (5) authentic UQ-9.

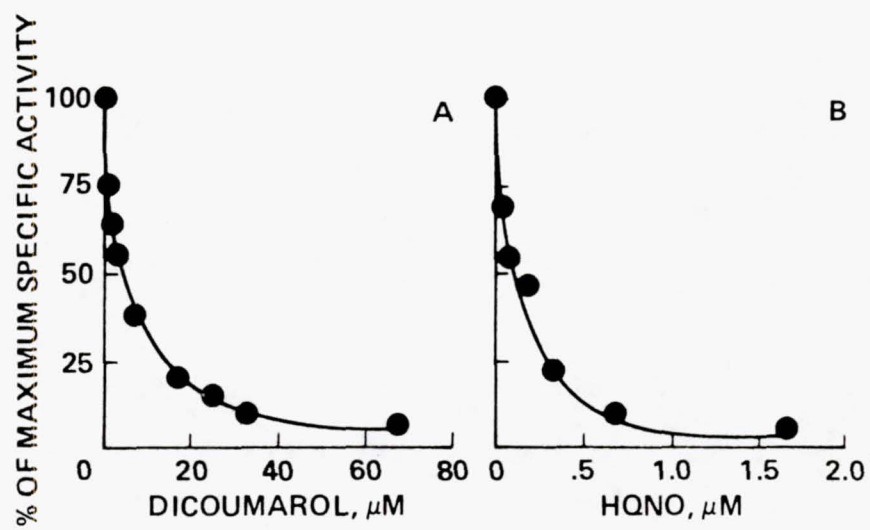


Fig. 1

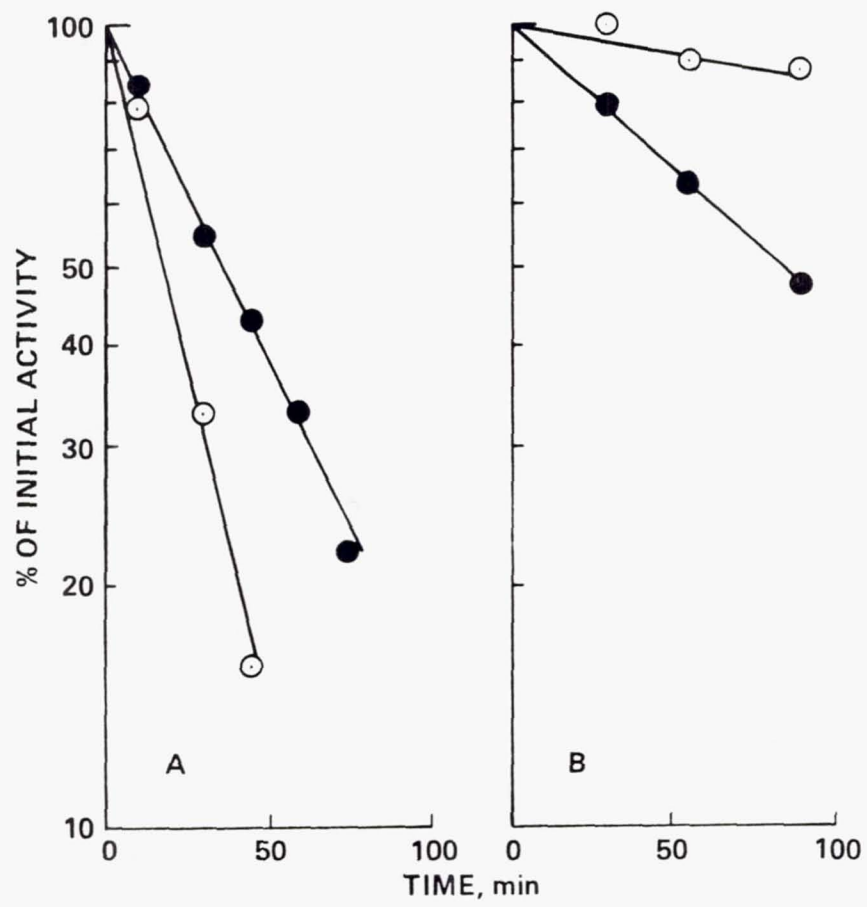


Fig. 2

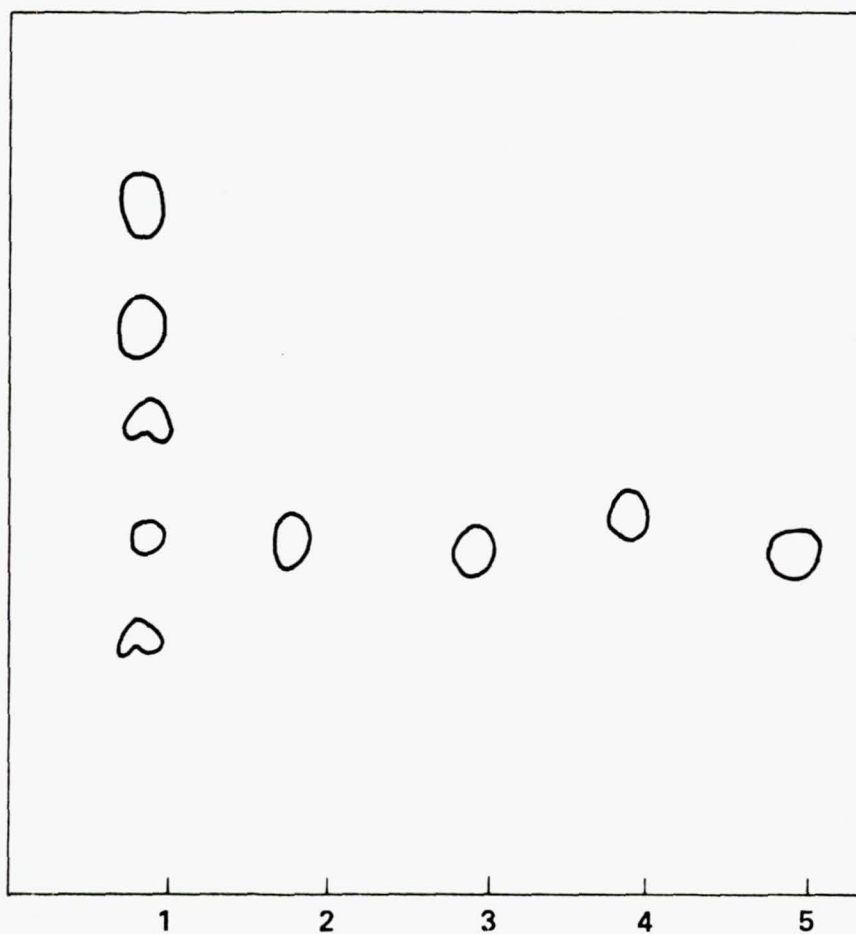


Fig. 3

1. Report No. NASA TM-84382		2. Government Accession No.		3. Recipient's Catalog No.	
4. Title and Subtitle ELECTRON TRANSPORT IN PARACOCCLUS HALODENITRIFICANS AND THE ROLE OF UBIQUINONE				5. Report Date June 1983	
				6. Performing Organization Code	
7. Author(s) Lawrence I. Hochstein and Sonja E. Cronin				8. Performing Organization Report No. A-9269	
9. Performing Organization Name and Address Ames Research Center Moffett Field, Calif. 94035				10. Work Unit No. T-4615	
				11. Contract or Grant No.	
12. Sponsoring Agency Name and Address National Aeronautics and Space Administration Washington, D.C. 20546				13. Type of Report and Period Covered Technical Memorandum	
				14. Sponsoring Agency Code 199-60-62-06-00	
15. Supplementary Notes Point of Contact: L. I. Hochstein, Ames Research Center, M/S 239-10, Moffett Field, Calif. 94035. (415) 965-5938 or FTS 448-5938.					
16. Abstract The membrane-bound NADH oxidase of <u>Paracoccus halodenitrificans</u> was inhibited by dicoumarol, 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO), and exposure to ultraviolet light (at 366 nm). When the membranes were extracted with n-pentane, NADH oxidase activity was lost. Partial restoration was achieved by adding the ubiquinone fraction extracted from the membranes. Succinate oxidation was not inhibited by dicoumarol or HQNO but was affected by ultraviolet irradiation or n-pentane extraction. However, the addition of the ubiquinone fraction to the n-pentane-extracted membranes did not restore enzyme activity. These observations suggested that reducing equivalents from succinate entered the respiratory chain on the oxygen side of the HQNO-sensitive site and probably did not proceed through a quinone.					
17. Key Words (Suggested by Author(s)) <u>Paracoccus halodenitrificans</u> Ubiquinone-9 NADH and succinate oxidation				18. Distribution Statement Unlimited Subject Category: 51	
19. Security Classif. (of this report) Unclassified		20. Security Classif. (of this page) Unclassified		21. No. of Pages 23	
				22. Price* A02	