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Restoration by Ubiquinone of *Azotobacter vinelandii* Reduced Nicotinamide Adenine Dinucleotide Oxidase Activity

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Extraction with pentane virtually abolished reduced nicotinamide adenine dinucleotide oxidase activity in small particles from *Azotobacter vinelandii*, but activity was largely restored by added ubiquinone.

Attempts to demonstrate a role for the native *Azotobacter vinelandii* ubiquinone-8 (Q-8) in reduced nicotinamide adenine dinucleotide (NADH) oxidation by restoring Q-8 to acetone-extracted particles have been unsuccessful (4, 8). Accordingly, a pentane extraction method (7) was employed as it has proved relatively innocuous to mitochondria. Assays for menaquinone were also conducted.

A cell paste of *A. vinelandii* strain 0, suspended in four volumes of 0.02 M sodium phosphate buffer (*p*H 7.4), was disrupted in a French pressure cell, and cellular debris was removed by centrifuging for 15 min at 12,000 × *g*. A small-particle fraction, enriched in NADH oxidase activity, sedimented between 35,000 × *g* for 30 min and 117,000 × *g* for 60 min. This fraction was lyophilized and extracted by shaking three successive times for 5 min with pentane at 0 C. Pentane was removed from the residue by evacuation at room temperature for 15 min (R. T. Swank, M.S. Thesis, University of Wisconsin, 1967). Particles so prepared and subsequently stored in 20 mM phosphate buffer (*p*H 7.4) at −20 C lost no activity in restoration experiments over a period of 4 days.

Crystalline Q-8 was prepared from *A. vinelandii* (2) and was added back to pentane-extracted particles in a volume of ethyl alcohol that did not exceed 4% of the assay volume. NADH-oxidase assays were performed in all-glass Gilson constant-pressure differential volumometers with 5 mM NADH, 0.2 mM ethylenediaminetetraacetate, and 20 mM phosphate buffer (*p*H 7.4) at 30 C. Ubiquinone or other materials added were preincubated in 0.75 ml with extracted particles (0.05 to 0.10 mg) for 10 min at 30 C with shaking; the reaction was started by tipping in 0.25 ml of NADH from the side arm. Bioassays for vitamin K were conducted after feeding dried isooctane extracts of broken (French pressure cell), lyophilized cells to deficient rats and recording prothrombin times 22 hr later.

If any menaquinone was present, it was present at a concentration of less than 2.5 nmoles of vitamin K_1 per g (dry weight) of *A. vinelandii* based on the lack of reduction of clotting times, when 2.0 g of extract was fed, and on the reduction of clotting time by 5.0 μg of vitamin K_1 (Table 1). However, Q-8 is reported to be present at 2.6 μmoles per g, dry weight (6). Other investigators (3) have been unable to detect menaquinone in *A. vinelandii* by chromatographic and spectral means.

Figure 1 illustrates the concomitant loss of ubiquinone and NADH oxidase activity upon successive extractions with pentane. Native Q-8 (Table 2) restored the specific activity of extracted particles about 20-fold to 60 to 70% of the unextracted value. The efficiency of restoration per unit of Q-8 was quite low; from 100 to 200 times the normal level of ubiquinone found in the particles was required for maximal enhancement. However, the restored activity appeared physiological, as it was completely inhibited by 1 mM KCN. Also, addition of Q-8 at these levels to unextracted particles did not enhance the oxidation rate; rather, it inhibited about 15%.

Another ubiquinone, synthetic hexahydro Q-4 (Table 3), restored pentane-extracted particles to about 80% of their original activity. Again, restored activity was completely sensitive to 1 mM KCN, and Q-4 did not enhance the activity of unextracted particles. Under identical experimental conditions, vitamin K_1 at concentrations of 0.05 to 0.2 mM did not restore NADH oxidase activity.

Menadione at 0.2 mM boosted the activity of

TABLE 1. *Bioassay of pentane extracts from A. vinelandii for menaquinone activity*

Treatment[a]	Clotting time (sec)[b]
Control	>50
1.0 g of extract	>50
2.0 g of extract	>50
Vitamin K_1, 5 μg	23.4
Vitamin K_1, 10 μg	15.2

[a] Extracts and vitamin K_1 were fed by stomach tube in 0.5 ml of ethyl alcohol plus corn oil.

[b] Plasma clotting times were recorded with a Fibrometer (BBL) and are average values for three experiments.

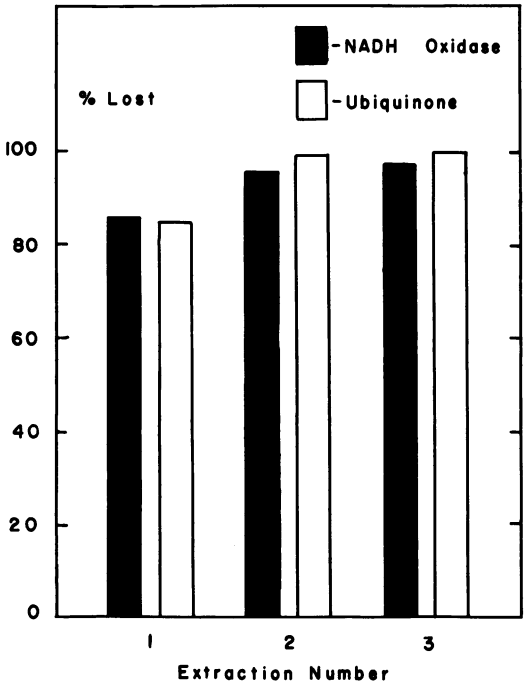

FIG. 1. *Loss of ubiquinone and NADH-oxidase activity from small particles of A. vinelandii upon extraction with pentane. The total ubiquinone removed by three extractions was 3.0 μmoles/g (dry weight) by spectrophotometric assay.*

extracted particles to 160% of the unextracted particle activity. However, the restored activity appeared nonphysiological, because this level of menadione also increased the NADH oxidase activity of unextracted particles by 95%, and 1 mM KCN inhibited only 7% of the oxidase activity restored by menadione. A similar lack of sensitivity to KCN was observed at *p*H 6.2, a *p*H at which menadione is not autoxidizable.

Desoxycholate, Triton X-100, Tween 80, and

TABLE 2. *Restoration by native ubiquinone-8 of NADH-oxidase activity in A. vinelandii particles extracted three times with pentane*

Ubiquinone-8 added	Specific activity[a]
mM	
None	2.06[b]
None	0.067
0.026	1.04
0.052	1.12
0.078	1.35
0.104	1.39

[a] Expressed as micromoles of O_2 per milligram of protein per minute.

[b] Unextracted control particles.

TABLE 3. *Restoration by hexahydrocoenzyme Q-4 of NADH-oxidase in particles extracted three times with pentane*

Hexahydro Q-4 added	Specific activity[a]
mM	
None	2.06[b]
None	0.067
0.033	0.764
0.065	1.01
0.130	1.26
0.260	1.51
0.375	1.48
0.630	1.67

[a] Expressed as micromoles of O_2 per milligram of protein per minute.

[b] Unextracted control particles.

a whole lipid extract from *A. vinelandii* added alone to extracted particles or in conjunction with the various quinones had no effect on the restoration of NADH-oxidase activity except to cause slight inhibitions.

NADH-oxidase activity was decreased precipitously by three successive extractions with acetone, and addition of 0.1 mM Q-8, in contrast to its effect on pentane-extracted particles, did not measurably restore activity. Apparently, acetone irreversibly damages a sensitive component in the terminal transport chain (1).

Recently Knowles and Redfearn (5) reported that succinate, L-malate, and NADH reduce up to 85% of endogenous ubiquinone of *A. vinelandii* particles as measured by extraction experiments, although the ubiquinone reductase rates were only 35 to 60% of the rates of oxidation of these substrates. Thus, it is possible that only a portion of the total ubiquinone, which is present in fourfold excess over other terminal electron com-

ponents in the small-particle fraction, is on the main pathway of electron transport. Our experimental results show clearly that ubiquinone-8 is an essential component of the NADH oxidase segment of terminal electron transport in *A. vinelandii*.

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