The NADH Dehydrogenase of the Respiratory Chain of
Escherichia coli

I. PROPERTIES OF THE MEMBRANE-BOUND ENZYME, ITS SOLUBILIZATION, AND PURIFICATION
TO NEAR HOMOGENEITY*

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The NADH dehydrogenase of the Escherichia coli respiratory chain has been identified by the
following properties: (a) its location in membrane vesicles; (b) its inhibition by AMP in a fashion similar
to that of the NADH oxidase; (c) its specificity for NADH, but not NADPH, with the same Kᵢ for
NADH as that of the NADH oxidase; (d) its sensitivity when membrane-bound to inhibition by
dicoumarol, rotenone, and 2-heptyl-4-hydroxyquinoline-N-oxide, which are also inhibitors of the NADH
oxidase. The NADH dehydrogenase of the cytosol fraction (assayed as NADH-dichlorophenolindophenol
reductase activity) differs substantially from the membrane-bound activity both in substrate specificity
and in the inhibitors of the reaction.

The respiratory chain NADH dehydrogenase was extracted from isolated membrane vesicle prepara-
tions by solubilization in Triton X-100, and was purified in buffers containing that detergent. The
purification employed chromatography on DEAE-cellulose, precipitation by 30% ethanol, and chroma-
tography on hydroxylapatite and DEAE-agarose. The most highly purified preparations of the enzyme
were homogeneous in migration on polyacrylamide gels containing Triton X-100, at pH 9.5, where one
band accounted for all of the protein and activity. Electrophoresis on polyacrylamide gels containing so-
dium dodecyl sulfate showed 1 band of molecular weight 38,000, which accounted for over 75% of the pro-
tein on the gel. Because of requirements for either Triton X-100 or phospholipid for activity of the purified
enzyme, it is difficult to estimate the level of purification achieved over isolated membrane vesicles.
However, we estimate that the enzyme was purified some 30-fold over membrane vesicles, or some
300-fold over whole cells.

Nicotinamide adenine dinucleotide (NAD⁺) has a pivotal role in metabolism as a carrier of reducing equivalents. NADH generated in catabolic pathways may participate in the synthe-
sis of anabolic intermediates, by transfer of its hydride ion to NADP⁺; alternatively, NADH may be used to generate meta-
bolic energy, by transfer of its electrons to an oxidant, via a chain of membrane-bound carriers. The entry of NADH into
this electron transport pathway is catalyzed by a respiratory chain-linked NADH dehydrogenase which commits the elec-
trons of NADH to the production of electrochemical gradients or ATP. Thus, this enzyme, located at a portal of the electron transport chain and catalyzing the oxidation of a critical cellular metabolite, is of central importance to the economy of the cell. For these reasons, we have begun a study of the

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The respiratory chain NADH dehydrogenase of E. coli has received less attention than its mitochondrial counterpart, which has been the subject of much research (1-18). Wosilait
and Nason (19) first reported on the catalytic properties of a particulate NADH-menadione reductase of E. coli, but made
no attempt to solubilize or purify the enzyme. Kashket and Brodie (20) reported the solubilization by deoxycholate of an
NADH-quinone reductase from a "small particle fraction" of E. coli. The enzyme, which was not purified, could oxidize
NADH (but not NADPH), was stimulated by FAD (but not FMN), and could reduce naphthoquinones and menadione. Solubilization resulted in a loss of 60% of the activity, and the
loss of activity did not appear to be due to inhibition by the deoxycholate.

Bragg (21) reported on the partial purification of an NADH-
menadione reductase from E. coli following solubilization of
cell extracts with deoxycholate. The enzyme oxidized both
NADH and NADPH, and was stimulated by both FAD and FMN. Further attempts to purify the deoxycholate-solubilized enzyme (22) gave three fractions with NADH dehydrogenase activity, one of which could reduce molecular oxygen directly. The three fractions had different catalytic properties with respect to substrate specificity, flavin requirement, inhibitor sensitivity, and electron acceptor specificity. The relationship between the fractions was not clarified, nor was any fraction identified as the respiratory chain-linked enzyme. Hendler and Burgess (23) also described multiple fractions with NADH dehydrogenase activity following deoxycholate solubilization of the particulate material obtained after sonication of spheroplasts of E. coli. At least three active fractions could be detected by hydroxylapatite and DEAE-cellulose chromatography. One of these, which could not be separated from the major portion of the succinate dehydrogenase activity, contained succinyl-CoA synthetase. The other two principal fractions of NADH dehydrogenase activity were separable from both succinate and d-lactate dehydrogenase, but both preparations contained multiple polypeptide components. One of these fractions appeared to be an aggregate, for it did not enter analytical disc gels at pH 9.5. No studies of the kinetic properties of the enzyme were reported, and the relationship between the different activities found was not explored further.

The appearance of multiple fractions of the deoxycholate-solubilized E. coli NADH dehydrogenase, and the variation in the enzymatic properties exhibited by the different enzyme preparations, suggest that the enzyme may be incompletely solubilized by this detergent and may also be altered by solubilization. It is also possible that the starting preparations were contaminated with soluble diaphorase activities which have been shown to exist in E. coli (20, 24). In this manuscript, we report on the identification and purification to near homogeneity of the respiratory chain-linked NADH dehydrogenase of E. coli. Subsequent communications will describe the kinetic and physical properties of the enzyme and its interaction with lipid.

MATERIALS AND METHODS

Materials—NADH, NADPH, FAD, dichlorophenolindophenol, menadione, rotenone, dicoumarol, 2-heptyl-4-hydroxyquinoline-N-oxide, and lysozyme were purchased from the Sigma Chemical Co.; Triton X-100, from Rohm and Haas; DEAE-cellulose, DEAE-agarose, hydroxylapatite, Bio-Gel A-1.5m, acrylamide, and N,N'-methylenbisacrylamide, from Bio-Rad Laboratories.

Growth of Escherichia coli—E. coli K12 strain CRT 4615 (Hfr, leu, thi, tnaA, str) was grown to late exponential phase at 37°C with vigorous aeration in a rich medium (ML) containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter or, alternatively, in a minimal medium (MOD 63) containing 12.5 g of K,HPO₄, 3.8 g of KH₂PO₄, 2 g of (NH₄)₂SO₄, 0.2 g of MgSO₄, 0.5 mg of FeSO₄, 5 mg of thiamin/Cl, and 20 mg of leucine per liter. The latter was supplemented with either glucose (0.2%), lactose (0.2%), succinate (0.3%), or lactate (0.3%) as the sole carbon and energy source. Cells were harvested at 0-4°C using a Sharples continuous flow centrifuge and were stored frozen at -75°C.

Membrane Vesicle Preparation—E. coli membrane vesicles were prepared as described by Kaback (25), with slight modification. In order to avoid the large volumes of material during centrifugation following the lysozyme/EDTA and spheroplast lysis steps. The volumes used at these steps were, respectively, 2- and 4-fold lower than those called for in the original procedure, and the lysozyme concentration was doubled.

Following spheroplast lysis, membrane vesicles were harvested in a Sorvall SS-14 continuous flow rotor at 17,000 rpm. The flow rate used with this rotor is critical. If the rate is too slow, the distributor of the rotor becomes obstructed by the membrane precipitate, whereas too high a flow rate results in a substantial loss in membranes, which pass through with the supernatant. A balance between these two effects was obtained at flow rates between 90 and 120 ml/min, and the maximum flow rate was checked for each preparation by reducing the flow rate until the optical density at 600 nm of the supernatant solution was between 10 and 20% of that of the original lysate. The membrane vesicle fraction recovered after lysis of spheroplasts was subjected to vigorous homogenization to ensure complete removal of cytoplasmic contaminants.

Enzyme Assays—Unless otherwise noted, all assays were performed as described in a Gilford 240 spectrophotometer equipped with a strip chart recorder. cuvettes with a 1-cm light path were used. NADH oxidase activity was measured by following the decrease in optical density at 340 nm. The assay mixture contained the following in a final volume of 1 ml: 0.1 M Tris/Cl (pH 7.5), 200 μM NADH, and enzyme. NADH DCIP reductase activity was measured by following the decrease in optical density at 600 nm. The assay mixture contained in a final volume of 1 ml: 0.1 M Tris/Cl (pH 7.5), 250 μM NADH, 10 mM KCN, 40 μM DCIP, and enzyme. Values were corrected for the reduction of DCIP which occurred in the absence of enzyme.²

NADH-menadione reductase activity was measured by following the decrease in optical density at 340 nm. The assay mixture contained the following in a final volume of 1 ml: 0.1 M Tris/Cl (pH 7.5), 150 μM NADH, 10 mM KCN, 20 μM FAD, 70 μM menadione, and enzyme. Values were corrected for the oxidation of NADH which occurred in the absence of menadione. NADH-ferricyanide reductase activity was assayed by following the decrease in optical density at 420 nm. The assay mixture contained in a final volume of 1 ml: 0.1 M Tris/Cl (pH 7.5), 250 μM NADH, 10 mM KCN, 40 μM FAD, 500 μM potassium ferricyanide, and enzyme.

When assaying the Triton-solubilized or the purified enzyme preparations, it is necessary to include Triton X-100 in the menadione, DCIP, and ferricyanide reductase assays. Due to the variation in Triton X-100 concentration during the course of enzyme purification, and also in different purified enzyme preparations, it is difficult to maintain a constant, noninhibitory concentration of Triton for all assays. Therefore, during the course of enzyme purification, the level of Triton X-100 in the assay mixture was always adjusted to 0.1%, which slightly inhibits the purified enzyme. For all other studies, unless otherwise noted, the concentration of Triton in the assay was adjusted to 0.04 to 0.06%.

Specific activities are expressed as units/mg of protein. One unit of enzyme activity is defined for the different activities as follows. NADH oxidase and menadione reductase, the amount of enzyme that results in the oxidation of 1 μmol of NADH/min; DCIP reductase, the amount of enzyme that results in the reduction of 1 μmol of DCIP/min; ferricyanide reductase, the amount of enzyme that results in the reduction of 1 μmol of ferricyanide/min. The following extinction coefficients were used in the calculations. NADH, 6.2 mM⁻¹ cm⁻¹ (26); DCIP, 20.5 mM⁻¹ cm⁻¹ (27); ferricyanide, 1 mM⁻¹ cm⁻¹ (28).

In describing the results, NADH-DCIP reductase and NADH dehydrogenase are used synonymously. In the cases where other electron acceptors are used, the enzyme is referred to as NADH (acceptor) reductase.

Polyacrylamide Gel Electrophoresis—Analytical disc gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Weber and Osborn (30) on 10 cm, 7% acrylamide, 0.5% N,N'-methylenebisacrylamide gels containing 0.1% sodium dodecyl sulfate. Following electrophoresis, the gels were stained for protein using Coomassie blue (31).

Polyacrylamide gel electrophoresis in the presence of Triton X-100 was performed on 4-cm gels using the pH 9.5 system of Davis (32). Separating gels were composed of 7% acrylamide, 0.25% bisacrylamide. In order to prevent aggregation of the enzyme during the run, Triton X-100 was incorporated into the stacking and separating gel buffers at a final concentration of 0.1%. At this concentration, Triton prevents the gels from adhering to the glass tubing; this made it easier to remove the gels from the tubes.

The abbreviations used are: DCIP, dichlorophenolindophenol; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; menadione, rotenone, dicoumarol, 2-heptyl-4-hydroxyquinoline-N-oxide.

²Dithiothreitol reduces DCIP nonenzymatically and can interfere with the enzyme assay at levels that give more than a 10% reduction of DCIP.
necessary to support the gel with a small piece of cheesecloth placed over the bottom of the gel tube. The cheesecloth was conveniently held in place with a short piece of rubber tubing. In order to preserve enzyme activity, electrophoresis was performed at 2-4°C and 2 mA/gel. Following electrophoresis, gels were either stained directly for protein using Coomassie blue (31), or were first stained for enzyme activity and then protein. For comparison of bands of enzyme activity and protein, fine copper wire was used to mark the position of the NADH dehydrogenase activity prior to Coomassie blue staining. The enzyme activity stain has the following composition: 0.2 M Tris/Cl (pH 7.5), 500 µM NADH, 100 µM FAD, 200 µM DCIP, 10 mM KCN, and 0.1% Triton X-100. The bands of activity, which appear as zones of decolorization, were most intense between 30 and 50 min following the addition of DCIP to start the reaction. After the gels were removed from the stain, the intensity of the bands diminished rapidly with diffusion of the reduced DCIP, making photography difficult.

RESULTS

Effect of Growth Medium on Activity

To identify the growth conditions which would give the highest activity of the membrane-bound NADH dehydrogenase, *Escherichia coli* was grown with several different carbon and energy sources in minimal medium, as well as in rich medium, and the NADH oxidase and NADH dehydrogenase activities were measured. Table I shows the activity of NADH oxidase and NADH dehydrogenase (as measured using DCIP as the electron acceptor) in the supernatant and crude membrane pellet obtained by centrifugation of sonicated cells. If one assumes that all NADH oxidase activity requires an intact electron-transport chain, then the oxidase activity in the supernatant reflects membrane material which was not pelleted under the conditions of centrifugation. The NADH oxidase or NADH dehydrogenase activity of the pellet fractions was greatest with cells grown on rich medium or on minimal medium with glucose or lactate as the sole carbon and energy source. Cells grown on succinate or lactate gave one-half to one-third as much membrane-bound oxidase and dehydrogenase activities.

Since *E. coli* cells grown in either rich medium or glucose-minimal medium gave equivalent high levels of NADH oxidase and dehydrogenase activities in the membrane pellet fraction, both growth conditions were used in enzyme purification. No major differences in the properties of the enzyme prepared from cells grown on either glucose or rich medium have been detected.

### Table I

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>NADH oxidase</th>
<th>NADH dehydrogenase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Pellet</td>
</tr>
<tr>
<td>Rich medium</td>
<td>0.092</td>
<td>0.82</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.087</td>
<td>0.83</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.049</td>
<td>0.75</td>
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<tr>
<td>Succinate</td>
<td>0.028</td>
<td>0.42</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.050</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*In all cases, the pellet fractions hold between 19 and 22% of the total amount of protein in the sonicated cell suspension.*

### Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Dehydrogenase</th>
<th>Oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- AMP</td>
<td>+ AMP</td>
</tr>
<tr>
<td>Supernatant</td>
<td>6.7</td>
<td>6.0</td>
</tr>
<tr>
<td>Pellet</td>
<td>4.8</td>
<td>0</td>
</tr>
</tbody>
</table>

### Relationship between NADH Dehydrogenase of Supernatant and Membrane Fractions

The data in Table I point out one of the difficulties encountered in the study of this enzyme. Even when a correction is made for the incomplete recovery of membranes in the pellet, as judged by the recovery of NADH oxidase, the total amount of NADH dehydrogenase activity associated with the membrane fraction constitutes only 10 to 20% of the total activity in whole cells. Similar results have been obtained by others, who assumed that the NADH dehydrogenase activity in the supernatant fraction is identical with that associated with the membrane, i.e. the electron transport chain-linked enzyme (23). It seems more likely that the activity of the supernatant is due to the presence of other flavoprotein dehydrogenases, such as dihydrolipoyl dehydrogenase (33, 34), which oxidize NADH in the presence of an electron acceptor such as DCIP, but which are not necessarily related to the electron transport chain-linked enzyme. Previous studies support the conclusion that such diaphorase activities exist in *E. coli* (20, 24).

The following findings support the hypothesis that the membrane-bound NADH dehydrogenase is distinct from that in the supernatant. (a) The NADH dehydrogenase and NADH oxidase which are associated with the particulate fraction from sonicated cells are completely inhibited by 10 mM AMP, whereas the NADH dehydrogenase of the supernatant is only inhibited about 10% at this same concentration of AMP (Table II). It seems unlikely that the lack of inhibition by AMP of the soluble activity is the result of an allotypic change in the enzyme, since, as shown below (Table V), the Triton-solubilized enzyme is inhibited by AMP and has the same Kₚ for AMP as does the membrane-bound enzyme. The slight inhibition by AMP of the supernatant activity could easily be accounted for by residual membrane still present in the supernatant, as discussed above. The NADH oxidase activities of the supernatant and pellet fractions were equally inhibited by AMP.
AMP (about 90%). (b) NADPH is oxidized by unfractionated E. coli extracts with DCIP as an electron acceptor, but is not oxidized by isolated membrane vesicles, which are specific for NADH oxidation (Table III). (c) When fractionated E. coli extracts are treated with Triton X-100 and electrophoresed on anionic disc gels in the presence of Triton X-100, staining of the gels for NADH dehydrogenase using DCIP as the electron acceptor, revealed the presence of several bands of NADH dehydrogenase activity (Fig. 1, gel 1). Isolated membrane vesicles, similarly solubilized in Triton X-100, possess only two enzyme species, which show up as relatively sharp bands when gels are stained for NADH dehydrogenase activity (Fig. 1, gel 2). The diffuse nature of the bands of activity seen on gels of unfractionated cell extracts indicates that several enzymes may be present in each band. As will be discussed in a future paper, one of the two enzyme fractions seen after electrophoresis of Triton-solubilized membrane preparations appears to be the result of the association of a low molecular weight component with the NADH dehydrogenase. (d) As shown below, solubilization of isolated membranes in Triton X-100 results in a 50 to 70% reduction in the NADH dehydrogenase activity, whereas treatment of unfractionated cell extracts with Triton X-100 results in only a 10 to 15% reduction in activity. Thus, the membrane-bound activity is quite sensitive to treatment with Triton X-100. The small decrease in activity in the unfractionated extracts can be attributed to the membrane-bound enzyme present. (e) The supernatant has a higher ratio of dehydrogenase to oxidase activity under all growth conditions (Table I) than does the pellet fraction, which suggests that a large proportion of the dehydrogenase activity in the supernatant is not associated with the electron transport chain. Taken together, the above data suggest that the particulate fraction of E. coli extracts contains a distinct NADH dehydrogenase. Thus, this enzyme is referred to as the membrane-bound NADH dehydrogenase, or respiratory chain NADH dehydrogenase, and is assumed to be the first enzyme in the NADH oxidase pathway. The above data also suggest that E. coli contains NADH diaphorase activities which are not related to the electron transport chain-linked NADH dehydrogenase. Because of the difficulty in distinguishing between several NADH dehydrogenase activities in unfractionated cell extracts, membrane vesicles prepared by osmotic shock were used as starting material for purification of the enzyme. Such membrane vesicles have been shown to be substantially free of cytoplasmic contamination (29).

**Enzyme Recovery in Membrane Vesicles Prepared by Osmotic Shock**

Table III shows the recovery of pyridine nucleotide dehydrogenase and oxidase activities in membrane vesicles prepared by osmotic shock. NADPH oxidation, using DCIP as an electron acceptor, was taken as a measure of cytoplasmic contamination and was never above 1% of that found in unfractionated cell sonicates. The specific activities of NADH dehydrogenase and NADH oxidase in isolated membrane vesicles were approximately half of those found for the particulate fraction obtained by centrifugation of whole cell sonicates (Table I).

**Triton X-100 Solubilization of NADH Dehydrogenase**

Several different detergents and chaotropic agents were investigated for effectiveness in solubilizing the membrane-bound NADH dehydrogenase. Triton X-100, sodium deoxycholate, BRIJ 58, guanidinium chloride, BRIJ 35, saponin, and sodium perchlorate were tested for their ability to solubilize the NADH dehydrogenase and preserve enzyme activity. Triton X-100 was the most effective agent in solubilizing and maintaining the activity of the enzyme and was chosen for further study. Fig. 2 shows the results of an experiment to determine the optimum concentration of Triton X-100 for solubilization of NADH dehydrogenase from membranes; 100% solubilization represents the solubilization of all of the activity that remains after disruption of the electron transport chain. The results show that at the protein concentration used, essentially complete solubilization of the enzyme is obtained between 0.5 and 1% Triton X-100 at 25°C. As seen in Fig. 2, EDTA had no effect on enzyme solubilization, although it was included in the purification procedure to avoid divalent cation-dependent aggregation of the enzyme. Complete solubilization of the enzyme by Triton X-100 occurs at temperatures between 16 and 20°C. Following solubilization, the temperature can be lowered without precipitation of the enzyme.

As noted above, the recovery of NADH dehydrogenase activity following Triton X-100 solubilization is only 30 to 50% of that of intact membrane vesicles assayed in the absence of Triton X-100. Although this may be due to a partial inactivation of the dehydrogenase itself, it may also be due to the nature of DCIP as an electron acceptor. If DCIP accepts electrons at several sites in the electron transport chain distal to the NADH dehydrogenase, and if NADH oxidation by this route is more efficient than direct reduction of DCIP by the primary dehydrogenase, the loss in activity with Triton solubilization may be the result of disruption of the electron transport chain. The data in Table IV, showing the effects of various electron transport inhibitors on the activity of NADH oxidase and the membrane-bound and Triton-solubilized

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1. G. F. Dancey and B. M. Shapiro, manuscript in preparation.
NADH dehydrogenase support the hypothesis that DCIP may accept electrons at several sites in the electron transport chain. The clearest results were those obtained with HQNO. Under conditions where HQNO gives almost complete inhibition of NADH oxidase (with no inhibition of the Triton-solubilized dehydrogenase), the membrane-bound NADH dehydrogenase is inhibited only about 50%. DCIP may be accepting electrons at several sites along the electron transport chain that are differentially affected by HQNO.

Fig. 3 shows a curve for the inhibition of HQNO in intact membranes. Low concentrations of HQNO result in a 40% inhibition, but much higher concentrations are needed to effect a further limited inhibition, again suggesting more than one site of action for HQNO. The effects of rotenone and diconamol are similar to those of HQNO, although diconamol significantly inhibits the solubilized NADH dehydrogenase. Gutman et al. (24) have shown a titration curve for diconamol which resembles that for HQNO in Fig. 3. Thus, it appears that at least two pathways exist for NADH-dependent DCIP reduction, one of which is more sensitive to electron transport inhibitors. The sensitive pathway disappears upon solubilization of the membrane with Triton X-100. The effects of HQNO indicate that this secondary pathway of NADH-dependent DCIP reduction may constitute as much as 50% of the DCIP reductase activity measured in intact membrane vesicles. The secondary pathway could account for a large portion of the loss in NADH dehydrogenase activity following solubilization of membranes with Triton X-100.

Antimycin A and dinitrophenol are only partially effective as inhibitors of either the membrane-bound or solubilized NADH dehydrogenase (Table IV), whereas they both inhibit the NADH oxidase more effectively. Two iron-chelating agents, O-phenanthroline and α,α′-bipyridyl, also were examined for inhibitory activity. At 1 mM, O-phenanthroline only slightly inhibited NADH oxidase and dehydrogenase and α,α′-bipyridyl was not effective.

Another indication that solubilization of NADH dehydrogenase with Triton X-100 does not result in a major alteration in enzyme structure leading to its inactivation is the similarity in the kinetic properties of the membrane-bound and solubilized enzyme, as shown in Table V. The Kᵣ for NADH is not altered significantly by solubilization, nor is the Kᵣ for AMP, a competitive inhibitor of the enzyme. Neither preparation will oxidize NADPH nor is either stimulated by FMN or FAD. The
Effects of different compounds on NADH oxidase and membrane-bound and Triton-solubilized NADH dehydrogenase

<table>
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<th>Compound</th>
<th>Concentration</th>
<th>Oxidase</th>
<th>Dehydrogenase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Membrane-bound</td>
<td>Solubilized</td>
</tr>
<tr>
<td>HQNO</td>
<td>5 µg/ml</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20 µg/ml</td>
<td>56</td>
<td>6</td>
</tr>
<tr>
<td>Dicoumarol</td>
<td>65 µM</td>
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<tr>
<td></td>
<td>130 µM</td>
<td>52</td>
<td>27</td>
</tr>
<tr>
<td>Rotenone</td>
<td>88 µM</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>166 µM</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>10 µg/ml</td>
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</tr>
<tr>
<td></td>
<td>20 µg/ml</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>34 µM</td>
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<td>6</td>
</tr>
<tr>
<td></td>
<td>68 µM</td>
<td>36</td>
<td>4</td>
</tr>
<tr>
<td>α-Phenanthroline</td>
<td>1 mM</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>α,α′-Bipyridyl</td>
<td>1 mM</td>
<td>4</td>
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a = data not obtained at stated concentration.

Enzymatic properties of NADH oxidase and membrane-bound and Triton-solubilized NADH dehydrogenase

<table>
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<tr>
<th>Property</th>
<th>Oxidase</th>
<th>Dehydrogenase</th>
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<tr>
<td></td>
<td>Membrane-bound</td>
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</tr>
<tr>
<td>Kₐ, NADH</td>
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<td>50</td>
</tr>
<tr>
<td>Kₐ, AMP</td>
<td>650</td>
<td>500</td>
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</table>

similarities in kinetic properties suggest that major allotypic changes do not occur upon solubilization and indicate that the solubilized NADH dehydrogenase is the electron transport chain-linked enzyme.

Purification of the Triton-solubilized NADH Dehydrogenase

All procedures were carried out at 4–5°C unless otherwise indicated.

Step 1: Growth of E. coli—E. coli K12, strain CRT 4615 was grown to late exponential phase at 37°C on rich medium. The cells were harvested using a Sharples continuous flow centrifuge and were stored frozen at −70°C, where the enzyme activity remained stable.

Step 2: Preparation of Membrane Vesicles—Frozen cells (100 to 120 g, wet weight) were thawed and washed once with 4 liters of 0.01 M Tris/Cl (pH 8.0) at 4°C. Membrane vesicles were then prepared as described under “Materials and Methods.” Membrane vesicles could be stored frozen at −75°C for several months with no loss of NADH dehydrogenase or oxidase activity.

Step 3: Triton X-100 Solubilization—Membrane vesicles were washed with 0.02 M Tris/Cl (pH 7.5) and then suspended in the same buffer at a final protein concentration of 10 mg/ml. The suspension was warmed to 20°C, and an equal volume of 10% Triton X-100, 0.02 M EDTA (pH 7.5) was added. After continuous stirring for 15 min, the suspension was cooled on ice to 4–5°C and then centrifuged at 45,000 × g for 60 min. The pellet was discarded, and 10 mM dithiothreitol was added to the supernatant, after removing a sample for determination of enzymatic activity.

Step 4: DEAE-cellulose Chromatography—The supernatant solution from the previous step was applied to a DEAE-cellulose column (2 x 60 cm) equilibrated with 10 mM Tris/Cl (pH 7.5) containing 1% Triton X-100, 0.5 mM EDTA, and 0.1 mM dithiothreitol (DEAE-buffer). The column was washed with 600 to 700 ml of DEAE-buffer and then developed with a 1600-ml linear sodium chloride gradient (0 to 0.4 M) in DEAE-buffer at a flow rate of 50 ml/h. The results of this procedure are shown in Fig. 4A. Any increase in the amount of material applied to this column without a proportional increase in the column’s dimensions resulted in overloading. Increasing the ratio of the bed volume to the sample volume did not improve the separation and usually resulted in a lower recovery of enzyme activity. Varying the steepness of the gradient had little effect on separation.

Step 5: Ethanol Precipitation—The fractions from the previous step that contained the peak of enzyme activity were pooled and concentrated 5-fold by Amicon ultrafiltration using an XM-50 membrane. Ethanol (90%) at −10°C was added slowly to a final concentration of 30% with continuous stirring; the temperature during addition was maintained below 2°C by cooling on an ice/water/NaCl bath (−8 to −10°C). The mixture was stirred for 15 min at −2 to −5°C and then centrifuged at 16,000 × g for 15 min at −5°C. The pellet was resuspended in one-half the original volume of 50 mM potassium phosphate (pH 7.0) containing 1% Triton X-100, and after an assay for enzyme activity, dithiothreitol was added to a final concentration of 0.1 mM. About 60% of the starting protein was recovered in the ethanol precipitate along with essentially all the starting enzyme activity (Table VI). Ethanol precipitation requires at least 20 mM sodium (or potassium) chloride, which is already present following DEAE-cellulose chromatography. With ethanol concentrations below 25% or sodium chloride concentrations between 0 and 10 mM, no enzyme activity and little protein was precipitated. This finding made the possibility of further purification by ethanol precipitation unlikely.

Step 6: Hydroxyapatite Chromatography—The solubilized ethanol precipitate was applied to an hydroxyapatite column (5 x 20 cm) equilibrated with 50 mM potassium phosphate (pH 7.0) containing 1% Triton X-100 and 0.1 mM dithiothreitol (HT buffer). The column was washed with 2 column volumes of HT buffer followed by elution with two-step gradients of potassium phosphate, at 0.2 M 0.5 M potassium phosphate, (pH 7.0) both containing 1% Triton and 0.1 mM dithiothreitol, at the flow rate of 100 ml/h. At least 2 column volumes were used for each step gradient elution. Fig. 4B shows the results of a typical run on this column. The major portion of enzyme was eluted at pH 7.5; at 4°C, the pH was 8.0.
in the 0.5 M step, although some enzyme occasionally appeared in the 0.2 M step.

Attempts to improve the purification with gradient steps between 0.2 and 0.5 M potassium phosphate eluted both enzyme and protein in significant amounts with no increase in specific activity. Elution with a linear phosphate gradient also did not improve the purification. Varying the size of the column affects the optimal concentrations of phosphate buffer in the step gradient elution. In particular, the 0.2 M potassium phosphate step must be decreased to 0.1 to 0.15 M, when the total column volume is reduced by one-half, in order to avoid substantial losses of enzyme. At present it is not clear whether changing the column size has a pronounced effect on the final purification achieved.

**Step 7: DEAE-Agarose Chromatography**—The fractions from the 0.5 M potassium phosphate wash of the previous step containing the major portion of activity were pooled and dialyzed against 10 mM potassium phosphate (pH 7.0) containing 0.2% Triton X-100 and 0.1 mM dithiothreitol. The dialyzed sample was then applied to a DEAE-agarose column (1.5 x 25 cm) equilibrated with the same buffer. After applying the sample, the column was washed with 50 ml of the buffer and then a 400-ml linear 0-0.1 M NaCl gradient in buffer was used to develop the column at a flow rate of 20 ml/h. The results of a typical run are shown in Fig. 4C. Fractions containing the major portion of activity were pooled and concentrated by Amicon ultrafiltration using an XM-50 membrane.

The enzyme can be stored for about 2 months at -80°C without significant loss in activity, provided that it is not subjected to repeated freezing and thawing.

**Results of Purification**

Table VI shows the results of a purification of NADH dehydrogenase following the procedure outlined above. The purification is shown in relation to Triton/EDTA-solubilized membrane vesicles. Comparison of the specific activity of purified enzyme with that of whole cell extracts is hampered by the presence in whole cells of NADH diaphorase activity which, as discussed above, probably is unrelated to the respiratory chain-linked enzyme. It also is difficult to compare the specific activity to that found in intact membranes since, as discussed above. Triton/EDTA solubilization results in a 50 to 70% loss in enzyme activity which is probably due to removal of other membrane components.

Based on the activity found in Triton/EDTA-solubilized membrane vesicles, an overall purification of 16-fold can be achieved with about an 18% recovery of activity. The recovery from each of the individual steps has been variable, with the exception of the ethanol precipitation step. DEAE-cellulose chromatography gave recoveries ranging from 50 to 75%; hydroxylapatite chromatography, 30 to 50%; DEAE-agarose chromatography, 50 to 75%. The overall purification achieved has varied from 9- to 16-fold from preparation to preparation. The specific activity of preparations showing lower levels of purification can be increased by passage over a second DEAE-agarose column under the same conditions; however, this causes a further 50% loss in total activity.

The figures noted in parentheses in Table VI are an upward correction of the final purification values based on the stimula-

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**Table VI**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Recovery</th>
<th>Specific activity</th>
<th>Purification</th>
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<tr>
<td>Triton solubilization</td>
<td>800</td>
<td>16.0</td>
<td>100</td>
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<td>DEAE-cellulose chromatography (pH 8.0)</td>
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<td>11.2</td>
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<td>Ethanol precipitation</td>
<td>128</td>
<td>11.2</td>
<td>70</td>
<td>0.087</td>
<td>4.3</td>
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<tr>
<td>Hydroxylapatite chromatography</td>
<td>30</td>
<td>3.6</td>
<td>23</td>
<td>0.12</td>
<td>6.0</td>
</tr>
<tr>
<td>DEAE-agarose chromatography (pH 7.0)</td>
<td>6.0</td>
<td>2.0</td>
<td>16</td>
<td>0.32</td>
<td>16.0</td>
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</tbody>
</table>

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Fig. 4. Chromatography of NADH dehydrogenase on hydroxylapatite, DEAE-cellulose, and DEAE-agarose. The details of the chromatography of NADH dehydrogenase on DEAE-cellulose (A), hydroxylapatite (B), and DEAE-agarose (C), are described in Steps 4, 6, and 7, respectively, of the purification given under “Results.” NADH dehydrogenase activity was assayed at a final Triton concentration of 0.10%. Fraction volumes in A, B, and C were 20, 20, and 4.5 ml, respectively. All columns were developed at 4°C.

The enzyme can be stored for about 2 months at -80°C without significant loss in activity, provided that it is not subjected to repeated freezing and thawing.

The figures noted in parentheses in Table VI are an upward correction of the final purification values based on the stimula-
Purification of the purified enzyme by the phospholipid cardiolipin, which will be discussed in a future manuscript. This effect is not seen with the crude enzyme, suggesting that phospholipids which are required for activity are removed during the course of purification.

Purified NADH dehydrogenase can also oxidize NADH if either menadione or ferricyanide is used as the electron acceptor. It was thus of interest to see whether these 2 activities co-purified with the DCIP reductase activity. Table VII shows the results of a purification in which the activities with each electron acceptor were followed. The recovery and purification at each step are similar, but not identical, for the three activities. The final purification for DCIP reductase is about 2 times greater than that for menadione or ferricyanide reductase. The reason for these slight differences with different electron acceptors is not known, but may relate to the association of the enzyme with other membrane components which affect the activities differentially and which are removed with purification. Also note that, whereas the activity in intact and Triton-solubilized vessels differs for the DCIP reductase, as discussed above, it is the same for the ferricyanide and menadione reductase reactions.

Sodium Dodecyl Sulfate-Disc Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of the denaturing detergent sodium dodecyl sulfate has been used to examine the purity of the enzyme. The results using the Weber-Osborn system (30) are shown in Fig. 5. Removal of Triton X-100 by precipitation of the preparations with acetone, ethanol, or trichloroacetic acid had no effect on the mobility of the enzyme. Gels 1 to 4 show the results of several preparations of the enzyme, employing modifications of the purification procedure outlined above. Gel 1 is of the most highly purified preparation of enzyme thus far obtained from cells grown in rich medium (see Table VI), and only a single major protein band is seen along with some minor contaminants. By comparison of the mobility of the major protein band with that of known standards, it is estimated to have a molecular weight of 38,000. Gel 2 is a second preparation of the enzyme also obtained from cells grown in rich medium and is included to demonstrate the variability in degree of purity obtained when the same purification protocol is followed. This second preparation is contaminated to a greater extent, especially by proteins of higher molecular weight. The major band present is, however, the same as that seen in Gel 1. Gel 3 shows the results of a purification of the enzyme from cells grown on glucose as the sole carbon and energy source. The degree of contamination is similar to that seen for the preparation from cells grown in rich medium shown in Gel 2. Gel 4 shows the results of a purification from membranes obtained from a sonicated suspension of cells by a 0 to 30% ammonium sulfate precipitation described by Kohn and Kaback (37). The protein responsible for the major band in Gel 1 appears to be only a minor component in the preparation of Gel 4. The final specific activity of the enzyme purified according to our protocol from membranes prepared in this manner is 7- to 8-fold less than that obtained from membrane vesicles prepared by osmotic shock.

TABLE VII

Comparison of purification of NADH dehydrogenase when dichlorophenolphthol, ferricyanide, and menadione are used as electron acceptors

Details of enzyme purification are given in text. Whole cell extract was prepared as described in Table III. Assays for NADH dehydrogenase using different electron acceptors are described under Materials and Methods. Whole cell extracts and membrane vesicles (sonicated) were assayed in the absence of Triton X-100. All other fractions were assayed at a final Triton X-100 concentration of 0.1%. Cells (190 g, wet weight) grown in glucose minimal media were used in the purification.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Electron acceptor</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dichlorophenolphthol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total activity</td>
<td>Purification</td>
<td>Total activity</td>
</tr>
<tr>
<td>Whole cell extract</td>
<td>5,437</td>
<td>36,300</td>
<td>2,516</td>
</tr>
<tr>
<td>Membrane vesicles</td>
<td>311</td>
<td>720</td>
<td>123</td>
</tr>
<tr>
<td>Triton solubilization</td>
<td>124</td>
<td>1,0</td>
<td>760</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>42,7</td>
<td>2,0</td>
<td>187</td>
</tr>
<tr>
<td>Ethanol precipitation</td>
<td>36,0</td>
<td>3,3</td>
<td>125</td>
</tr>
<tr>
<td>Hydroxylapatite chromatography</td>
<td>14,6</td>
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<td>45</td>
</tr>
<tr>
<td>DEAE-agarose chromatography</td>
<td>8,73</td>
<td>9,5</td>
<td>28</td>
</tr>
</tbody>
</table>

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified NADH dehydrogenase. Polyacrylamide gel electrophoresis in 7% acrylamide, 0.25% bisacrylamide gels containing sodium dodecyl sulfate was performed as described under Materials and Methods. Gels are of: (I) 30 μg of purified enzyme from rich medium-grown cells (see Table VI); (2) 25 μg of purified enzyme from rich medium-grown cells (different preparation from that shown in Gel 1); (3) 30 μg of purified enzyme from glucose-grown cells; (4) 30 μg of an enzyme preparation from membranes prepared by NH₄SO₄ precipitation of cell sonicates (30); (5) 100 μg of membrane protein from rich medium-grown cells; (6) 100 μg of membrane protein from glucose-grown cells. See text for discussion of band marked with arrow.
Gels 5 and 6 of Fig. 5 show the proteins found in unfraccionated membranes from rich medium- and glucose-grown cells, respectively. The strong contaminant seen in less pure preparations of enzyme from rich medium-grown cells (Gel 2) correlates with a relatively intense band found in the corresponding membranes (Gel 5). This band, which is marked with an arrow, is less intense in the membrane gel for glucose-grown cells (Gel 6) and also in the gel for partially purified enzyme from these same membranes (Gel 3). This contaminant has a molecular weight of about 65,000. This same enzyme preparation (shown in Gel 3) had a peak in its absorption spectrum which indicates a minor contamination by cytochrome b₁ of about 5% (data not shown). Because cytochrome b₁ has a reported minimum molecular weight of 62,000 to 66,000 (38), the band may represent that cytochrome.

The denatured enzyme has a mobility comparable to that of the major, relatively broad band seen in the gels of total membranes. This broad band can be resolved into a doublet when less protein is applied to the gels, and the enzyme corresponds to the slower moving member of this doublet. An estimate of the purity of the enzyme from densitometric measurements (data not shown) indicated that the enzyme represented about 35 to 75% of the protein present in different preparations of the enzyme. In Gel 1 of Fig. 5, it corresponds to 75% of the stained material. Despite the variability in purification, no significant differences in the enzymatic properties has been observed with preparations over this range of purification.

Anionic Disc Gel Electrophoresis

Polyacrylamide gel electrophoresis at pH 9.5 (32) in the presence of Triton X-100 has also been used to evaluate the purity of the enzyme, and the results are shown in Fig. 6. Gel 1 shows the results obtained with the most highly purified preparation of enzyme thus far obtained and represents the material run on Gel 1, Fig. 5. Only a single protein band enters the gel. Although there does appear to be material at the interface between the stacking and running gels, it is difficult to assess whether this indicates the presence of contaminants or is the result of precipitation of the enzyme during the initial stacking on these gels.

Triton Gel 2 shows the results of electrophoresis of a less purified preparation of the enzyme and corresponds to Gel 3, Fig. 5. A substantial amount of contamination exists in this preparation. The mobility of the enzyme on anionic gels run in the presence of Triton X-100 varies depending on the amount of protein applied. The best method for determining which protein band corresponds to the enzyme is to stain the gel for enzyme activity before staining for protein. The arrows in Fig. 6 indicate the protein bands that showed enzymatic activity.

**DISCUSSION**

We elected to begin our purification of the NADH dehydrogenase with membrane vesicles, since several NADH dehydrogenase activities exist in E. coli (Refs. 20-24; Fig. 1), and we were interested in isolating the enzyme which was associated with the respiratory chain. Membrane vesicles have only two bands of NADH dehydrogenase activity whereas the cytosol fraction has many such components (Fig. 1). The relationship between the two bands of active components in membrane vesicles is currently being studied (see below). The membrane-bound NADH dehydrogenase differs from the soluble enzyme in its association with the NADH oxidase activity of the cell (Table I), its inhibition by AMP (Table II), and its inability to use NADPH (Table III). It is similar to the NADH oxidase in its Kₘ for NADH and its Kₘ for AMP (Table V). For these reasons, it appears that the NADH dehydrogenase we isolated is indeed the respiratory chain-linked enzyme, and is the first enzyme in the NADH oxidase pathway of electron transport. This conclusion is substantiated by finding that antiserum directed against the purified enzyme inhibits NADH oxidase and NADH dehydrogenase activities equivalently (39). We are presently investigating the relationship between the NADH dehydrogenase which we have isolated and the soluble NADH dehydrogenase(s) of the E. coli cell.

When the purified preparation is examined by anionic polyacrylamide gel electrophoresis in Triton X-100-containing gels (Fig. 6, Gel 1) or by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Fig. 5, Gel 1) the enzyme is seen to be essentially homogeneous. One can estimate the purification obtained over whole cells by assuming that the isolated membrane fraction contains about 10% of the cell protein (25). Thus, the 30-fold purification (under optimal conditions of lipid activation) corresponds to a 300-fold purification over whole cells. Since the enzyme in the most highly purified preparation accounts for over 75% of the protein, we estimate that the NADH dehydrogenase makes up some 0.2% of the E. coli cell protein or about 2% of the protein of the membrane. The enzyme has been purified using conventional techniques, the only difference being the inclusion of Triton X-100 in all of
the buffers. The enzyme requires Triton X-100 in order to remain soluble. We have never obtained an active preparation of the purified enzyme unless we included either Triton X-100 or phospholipids, one of which is required for activity. Solubilization in Triton X-100 leads to no changes in the $K_m$ for NADH or the $K_m$ for AMP (Table V). The constancy of these kinetic parameters upon solubilization with Triton X-100 is in contrast to the case of solubilization with deoxycholate (21) which causes a change in the $K_m$ for NADH. The purification of deoxycholate-solubilized enzyme (21) led to the preparation of an NADH-menadione reductase that oxidized both NADH and NADPH and was soluble in the absence of detergent.

The activity with NADPH is a property that we have found to be associated with the soluble forms of the enzyme but not with the membrane-bound dehydrogenase (Table III). Thus, the purification of an activity from deoxycholate-solubilized cell extracts may have led to enrichment of both soluble and membrane-bound NADH dehydrogenases. As mentioned in the introduction, in the few cases where independent criteria have been used to estimate the purity of NADH dehydrogenase preparations from E. coli (21–24), multiple protein components have been seen in the most purified fractions.

The NADH dehydrogenase of the inner mitochondrial membrane has been purified in two different forms. Initial purification attempts, which used acid and ethanol extraction procedures, led to a low molecular weight form of the enzyme (1–5). On the other hand, a high molecular weight form of the enzyme was obtained when Triton X-100 (12) was used for the extraction. The two forms of enzyme differ significantly in physical and kinetic properties (14). However, the high molecular weight form can be converted to the low molecular weight form by the acid/ethanol extraction conditions used for the isolation of the low molecular weight form (10, 15). This finding has led to the hypothesis (8) that the high molecular weight mitochondrial NADH dehydrogenase is a multi-enzyme complex that contains the low molecular weight enzyme and additional components. In a similar fashion, we have seen two bands of NADH dehydrogenase activity when Triton-solubilized membrane preparations are separated by anionic disc gel electrophoresis (Fig. 1). The final form of the enzyme we have purified is composed of only one band (Fig. 6) when examined in the same fashion. From preliminary experiments using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, it appears that the two bands both contain the 38,000-dalton NADH dehydrogenase, but one contains an additional component. Thus, the catalytic activity seems to reside in the 38,000-dalton component which is the dominant species in the highly purified enzyme. We are interested in whether kinetic differences exist between the two NADH dehydrogenase activities in E. coli membrane vesicles and are now modifying the purification to obtain both components for further study. In addition, we are continuing to examine the physical and kinetic properties of the respiratory chain NADH dehydrogenase in order to see how it interacts with the remainder of the electron transport chain and how it participates in the regulation of energy metabolism in E. coli.

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