The NADH Dehydrogenase of the Respiratory Chain of Escherichia coli

II. KINETICS OF THE PURIFIED ENZYME AND THE EFFECTS OF ANTIBODIES ELICITED AGAINST IT ON MEMBRANE-BOUND AND FREE ENZYME*

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The purified respiratory chain NADH dehydrogenase of Escherichia coli oxidizes NADH with either dichlorophenolindophenol (DCIP), ferricyanide, or menadione as electron acceptors, with ferricyanide being oxidized at the highest rate. The apparent $K_m$ values for NADH are similar with the three electron acceptors (approximately 50 $\mu$M). The purified enzyme contains no flavin and has an absolute requirement for FAD, with $K_m$ values around 4 $\mu$M. The pH optimum of the enzyme appears to be between 6.5 and 7; the optimum is difficult to establish because of nonenzymatic reduction of DCIP at the lower pH values. Potassium cyanide stimulates the DCIP reductase activity about 2-fold, but has no effect on ferricyanide reductase. The enzyme exhibits hyperbolic kinetics with respect to NADH concentration in both the ferricyanide and DCIP reductase assays, but cooperativity is seen in the menadione reductase reaction. NAD$^+$ is an effective competitive inhibitor of the reaction ($K_i = 20$ $\mu$M); in the presence of NAD$^+$, the NADH saturation curve becomes cooperative, even in the DCIP reductase assay. Many adenine containing nucleotides are competitive inhibitors of the enzyme. The apparent $K_i$ values for these nucleotides as inhibitors of the purified enzyme, the membrane-bound NADH dehydrogenase, and the NADH oxidase are equivalent. An examination of inhibitory effects of a series of adenine nucleotides suggests that the inhibitors act as analogues of NAD$^+$, which is the true physiological inhibitor. The results suggest that the enzyme in situ is always partially inhibited by the levels of NAD$^+$ in the E. coli cell, and thus behaves in a cooperative fashion to changes in the NAD$^+$/NADH ratio.

An antibody has been elicited against the purified NADH dehydrogenase. Immunodiffusion and crossed immunoelectrophoresis show that the antibody is directed principally against the NADH dehydrogenase, with some activity against minor contaminants in the purified preparation. The antibody inhibits NADH dehydrogenase activity 50% at saturating levels. When this antibody preparation is used to examine solubilized membrane preparations, two major immunoprecipitates are found. A parallel inhibition of the membrane-bound NADH dehydrogenase and NADH oxidase activities is seen, supporting the hypothesis that the purified enzyme is indeed a component of the respiratory chain-dependent NADH oxidase pathway.

The respiratory chain NADH dehydrogenase of Escherichia coli has been purified and shown to be an essentially homogeneous protein, after gel electrophoresis in the presence of Triton X-100, which it requires for activity and solubility (1). When the enzyme is disaggregated and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, over 75% of the protein migrates as a component of molecular weight 38,000. Thus, the enzyme solubilized from E. coli membrane vesicles and purified in Triton X-100-containing buffers is composed principally of a single polypeptide component. The isolated enzyme has many of the properties of the membrane-bound NADH dehydrogenase and the NADH oxidase (1). This contrasts with the case of the mitochondrial NADH dehydrogenase. Isolation of a low molecular weight form of the mitochondrial enzyme leads to marked alterations in kinetic properties (reviewed in Ref. 2) in comparison with those of a more complex, high molecular weight form, or those of the membrane-bound enzyme. In this manuscript, we explore the kinetic properties of the purified enzyme, in order to compare it with the high and low molecular weight forms of the mitochondrial NADH dehydrogenase.

Since NADH occupies a critical position in metabolism, linking and catabolic and anabolic pathways, the flux of
NADH should be strictly regulated. Thus, the use of the reducing equivalents of NADH for the generation of energy, via the electron transport chain, or conversely, for the synthesis of new metabolites, after transhydrogenation to NADPH, might reduce NADH for the generation of energy. For this reason, we have explored the effects of metabolic inhibitors upon the purified respiratory chain NADH dehydrogenase as well as upon the enzyme in situ, in membrane vesicles.

In an attempt to define more clearly the role of the enzyme in membrane-dependent processes, we have obtained antibodies against the purified NADH dehydrogenase. The high specificity of the antibodies has been confirmed by immunodiffusion and crossed immunoelectrophoresis. A study of the effects of the antibody preparation on the activity of purified and membrane-bound enzyme forms supports the hypothesis (1) that the isolated enzyme is a component of the respiratory chain NADH oxidase.

MATERIALS AND METHODS

Enzyme Preparations and Assays—The preparation of membrane vesicles, Triton-solubilized NADH dehydrogenase, and purified NADH dehydrogenase has been described (1). Two independently isolated purified enzyme preparations were used in the kinetic analyses. These preparations were 35 and 75% homogeneous, respectively, as estimated by electrophoresis in gels containing Trition X-100, and gels containing sodium dodecyl sulfate (1). The two preparations behaved identically for all criteria examined. The NADH-dependent reduction of dichlorophenolindophenol, menadione, and ferricyanide were determined spectrophotometrically as previously described (1), as were the activities of the membrane-bound NADH dehydrogenase and NADH oxidase. The composition of the assay mixtures and any modifications are listed in the legends to the tables or the figures. Double reciprocal plots (3) were used to estimate apparent $K_m$ and $V_{max}$ values. When linear relationships were obtained, only the $K_m$ and $V_{max}$ ave presented; when plots of $1/v$ versus $1/s$ were nonlinear, the data are presented in the figures. Apparent $K_m$ values were obtained by the method of Dixon (4).

Flavin Analysis—The flavin content of the enzyme was estimated by absorption spectroscopy and by fluorometry (5). Absorption spectra were determined in a Cary model 15 recording spectrophotometer. Corrections were made for light scattering resulting from the presence of Triton micelles. Fluorometric determination of flavin content was performed by adding ice cold, 50% trichloroacetic acid to 2 ml of enzyme solution, containing 25 to 100 $\mu$g of enzyme protein and 5 mg/ml of bovine serum albumin, to give a final trichloroacetic acid concentration of 5%. Precipitated protein was removed by centrifugation for 10 min at 20,000 $\times$ g, and the supernatant solution was extracted three times with 3 volumes of chloroform and, finally, once with 3 volumes of ether. Standard FAD solutions were treated in a similar manner.

Preparation of Antiserum Against Purified NADH Dehydrogenase—A female New Zealand white rabbit weighing about 5 kg was used in the preparation of antiserum. Due to the limited amount of highly purified enzyme (estimated to be 75% pure) available for use in immunization, only a single rabbit was used as a source of antiserum. Before immunization, serum was taken as a nonimmune control. For the initial injection, an emulsion consisting of 0.5 ml of enzyme solution (500 $\mu$g of protein) and 0.5 ml of complete Freund’s adjuvant was injected subcutaneously in the scapular region. This was followed 20 days later by an intravenous injection of 200 $\mu$g of enzyme in 0.2 ml of saline (0.9% NaCl solution). Thereafter, 100 $\mu$g of enzyme was injected intravenously at 8- to 10-day intervals. Blood was taken from the ear vein 30 days following the initial injection and then at 10 day intervals, just prior to reimmunization with antigen. The antiserum was obtained by centrifugation (5,000 $\times$ g for 20 min) of the clotted blood sample.

Both control and anti-NADH dehydrogenase sera were found to interfere with enzyme activity measurements. A substantial portion of this interference could be eliminated by the preparation of a crude $\gamma$-globulin fraction. This was prepared by three successive, 0 to 50% ammonium sulfate precipitations (7). The (NH$_4$)$_2$SO$_4$ precipitates were centrifuged and resuspended in the original volume of 10 mM potassium phosphate (pH 7.2), 0.15 mM NaCl (phosphate-buffered saline buffer). After the third precipitation, the immunoglobulin fraction was dialyzed against phosphate-buffered saline buffer. The crude $\gamma$-globulin fraction was stored frozen at −20°C in small aliquots, which were thawed as needed.

Although the crude $\gamma$-globulin fraction was used in several initial studies of the inhibition of the enzyme by antibodies, it was found that further purification of the $\gamma$-globulin fraction with DEAE-cellulose (8) effectively eliminated almost all the nonspecific inhibition of enzyme activity. For this, 5 to 10 ml of the crude $\gamma$-globulin fraction (40 mg/ml of protein), previously dialyzed against column buffer was applied to a DEAE-cellulose column (1.5 × 25 cm), equilibrated with 10 mM potassium phosphate, (pH 7.6), 15 mM NaCl. The material which did not bind to the column was concentrated to the original, applied volume by Amicon ultrafiltration using a PM-10 membrane. The majority of antibody and about one-third of the applied protein was recovered in this fraction, which will be referred to as “DEAE-cellulose purified antibodies.”

Analysis of Antiserum Complexity—Qualitative analysis of the antisera prepared against the purified enzyme was done using the Ouchterlony, double immunodiffusion technique (9), and the Laurell, two-dimensional crossed immunoelectrophoresis technique (10). Immunodiffusion was performed at 30°C for 12 to 18 h on microscope slides coated with 1.5 to 2.0 ml of 1% agarose in phosphate-buffered saline buffer containing 0.1% Triton X-100. The Triton was required in the immunodiffusion plate. In its absence, the enzyme precipitated as it diffused out of the antigen well. Triton X-100 was also used during electrophoresis, which was performed on glass slides (5 × 7.5 cm) coated with 1% agarose, in 0.05 M barbital buffer (pH 8.6) containing 0.1% Triton X-100. The two-dimensional technique described by Laurell (10) involves first separating the mixture of antigens by electrophoresis in the absence of antiserum. Following this, antibody-containing agarose layers are used to drive the antigens into the antiserum-containing agarose layer, where precipitation occurs. Antibodies do not migrate in the electric field at this pH. After the reaction with antiserum was completed the immunoelectrophoresis and immunodiffusion plates were soaked for 2 days in 0.15 M NaCl, and then immunoprecipitates were stained for protein using Coomassie brilliant blue (11). Alternatively, plates were stained for carbohydrate using the Schiff reagent (12).

Acid extraction of FAD (6) was performed by adding ice cold, 50% trichloroacetic acid to 2 ml of enzyme solution, containing 25 to 100 $\mu$g of enzyme protein and 5 mg/ml of bovine serum albumin, to give a final trichloroacetic acid concentration of 5%. Precipitated protein was removed by centrifugation for 10 min at 20,000 $\times$ g, and the supernatant solution was extracted three times with 3 volumes of chloroform and, finally, once with 3 volumes of ether. Standard FAD solutions were treated in a similar manner.

Electron Acceptor Specificity—Purified NADH dehydrogenase can oxidize NADH with either dichlorophenolindophenol, ferricyanide, or menadione as electron acceptor. Saturation curves and double reciprocal plots of velocity versus acceptor concentration for DCIP and ferricyanide are shown in Figs. 1 and 2, respectively. Both of these acceptors inhibit the enzyme at high concentrations. Menadione does not inhibit at concentrations as high as 150 $\mu$M, 10 times the apparent $K_m$ for this acceptor (data not shown).

From double reciprocal plots of the saturation curves for these electron acceptors, the apparent $K_m$ and $V_{max}$ for each electron acceptor has been estimated, and these values are listed in Table I. The enzyme has a lower apparent $K_m$ for both DCIP and menadione than for ferricyanide, which is required at 40 times the concentration of DCIP to achieve half-maximal dehydrogenase activity. The value of $V_{max}$ obtained using

$^1$The abbreviation used is: DCIP, dichlorophenolindophenol.
The NADH-DCIP reductase activity of purified enzyme was determined as described in Ref. 1. The assay mixture contained in 1 ml: 0.1 M Tris/Cl (pH 7.5); 250 μM NADH; 10 mM KCN; 100 μM FAD; 0.05% Triton X-100; enzyme (15 μg); and the quantities of DCIP shown. Velocity (v) is given as units/mg of protein.

The NADH-ferricyanide reductase activity was determined as described in Ref. 1. The assay mixture contained the following, in 1 ml: 0.1 M Tris/Cl, pH 7.5; 250 μM NADH; 100 mM KCN; 100 μM FAD; 0.05% Triton X-100; enzyme (15 μg); and the quantities of ferricyanide shown. Velocity (v) is given as units/mg of protein.

The Hill coefficient determined at NADH concentration, as well as by inhibition at high concentrations of NADH, or FAD, respectively. Estimation of the Km, Vmax, and Vmax/Km of NADH for the crude, Triton X-100-solubilized enzyme is activated up to 30% by FAD, depending on the preparation. The purified enzyme has an absolute requirement for FAD. This requirement is maintained with all three electron acceptors. Saturation curves with FAD show hyperbolic kinetics; the apparent Km, for FAD is essentially the same for all three electron acceptors (Table II).

NADPH is not oxidized by the purified enzyme, nor do isolated membranes possess substantial NADPH oxidase or dehydrogenase activity (1). NADPH is also not oxidized with any of the three electron acceptors when FAD is replaced by FMN in the reaction mixture.

Co-factor Specificity—Whereas NADH oxidase and membrane-bound NADH dehydrogenase are not activated by FAD, the crude, Triton X-100-solubilized enzyme is activated up to 30% by FAD, depending on the preparation. The purified enzyme has an absolute requirement for FAD. This requirement is maintained with all three electron acceptors. Saturation curves with FAD show hyperbolic kinetics; the apparent Km, for FAD is essentially the same for all three electron acceptors (Table II). FMN cannot substitute for FAD in any of the assays. However, FMN does appear to bind to the enzyme since it inhibits the stimulation by FAD (data not shown).

Highly purified preparations of the enzyme do not absorb significantly in the 350 to 600 nm region, even at protein concentrations as high as 0.5 mg/ml, indicating that the purified enzyme does not contain flavin. In addition, no flavin was detected by fluorometric analysis after acid extraction of the enzyme (see “Materials and Methods”).

Effect on pH on Enzyme Activity—As shown in Fig. 4, both the membrane-bound and purified NADH dehydrogenases are maximally active at a pH between 6.5 and 7.0, when DCIP is used as the electron acceptor. Changing the buffer from imidazole to Tris/acetate had only a slight effect on enzyme activity. The activity of the purified enzyme was the same as that obtained in Tris/acetate buffer when measured at pH 7.4 in either potassium phosphate or Tris/Cl buffer. Measurement

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**Table I**

**Kinetic properties of purified NADH dehydrogenase**

All assays were performed as described under “Materials and Methods.” The composition of the DCIP reductase assay mixture was as in Fig. 1; that of the ferricyanide reductase assay mixture, as in Fig. 2; that of the menadione reductase assay mixture, as in Fig. 3. For each Km, determination, the concentration of either the electron acceptor, NADH, or FAD, respectively, was varied. Km, and Vmax values were determined from Lineweaver-Burk plots (3). The apparent Km, of NADH is essentially the same in the ferricyanide and DCIP reductase assays (Table I). The apparent Km, of NADH for the crude, Triton X-100-solubilized enzyme is activated up to 30% by FAD, depending on the preparation. The purified enzyme has an absolute requirement for FAD. This requirement is maintained with all three electron acceptors. Saturation curves with FAD show hyperbolic kinetics; the apparent Km, for FAD is essentially the same for all three electron acceptors (Table II).

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Inhibition of purified and membrane-bound NADH dehydrogenase by adenine nucleotides

The assay mixture for NADH-DCIP reductase was the same as that in Fig. 1, with 40 μM DCIP. The assay mixture for NADH oxidase contained 0.1 M Tris/Cl (pH 7.5), 200 μM NADH, 75 μg of enzyme. Oxidase and membrane-bound dehydrogenase were assayed in the absence of Triton X-100. Purified dehydrogenase was assayed in 0.05% Triton X-100. Membrane-bound dehydrogenase and oxidase activity was measured in membrane vesicles prepared as described in Ref. 1. Brief sonication (1 min) was performed on vesicles prior to assays. The apparent Kᵢ for nucleotides was determined by the method of Dixon (4).

![Graph](http://www.jbc.org/)

**Table II**

<table>
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<tr>
<th>Nucleotide</th>
<th>NADH dehydrogenase</th>
<th>NADH oxidase</th>
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<tbody>
<tr>
<td></td>
<td>Purified</td>
<td>Membrane-Bound</td>
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<tr>
<td>AMP</td>
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<td>0.6</td>
</tr>
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</tr>
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**Table III**

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<tr>
<td>Deoxyadenosine</td>
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<td>30</td>
</tr>
<tr>
<td>Deoxy-AMP</td>
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<td>41</td>
</tr>
<tr>
<td>Cyclic AMP</td>
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<td>76</td>
</tr>
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<tr>
<td>ATP</td>
<td>10</td>
<td>50</td>
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</tbody>
</table>

Inhibition of NADH dehydrogenase by adenine compounds

NADH-DCIP reductase was assayed as described under "Materials and Methods," with the assay mixture described in Fig. 1, and 40 μM DCIP. The inhibition at each NADH concentration is given as a percentage, in comparison with the activity measured in the absence of inhibitor. The specific activity of the preparation was 0.28 and 0.13 units/mg of protein at 250 and 32 μM NADH, respectively.

![Graph](http://www.jbc.org/)

**Effect of KCN on Enzyme Activity Using DCIP as Electron Acceptor**—Potassium cyanide at 5 to 10 mM stimulates NADH dehydrogenase activity almost 2-fold when DCIP is used as an electron acceptor (data not shown). This stimulation is not due to the presence of cyanide-inhibitable NADH oxidase activity in the enzyme preparation. Cyanide does not appear to act as a non-specific chelating agent, since at 5 mM levels sodium azide, guanidine hydrochloride, sodium perchlorate, and urea have no effect on activity. Additionally, cyanide does not stimulate enzyme activity when ferricyanide is used as an electron acceptor, suggesting that the effect involves the interaction of DCIP with the enzyme.

**Inhibitors of the Enzyme—NAD⁺, a product of the reaction catalyzed by NADH dehydrogenase, acts as a competitive inhibitor of NADH oxidation. This is shown in Fig. 5, which is a double reciprocal plot of velocity versus NADH concentration at different NAD⁺ concentrations.** In the absence of NAD⁺, the NADH saturation obeys Michaelis-Menten kinetics, with an experimental error. However, as the NAD⁺ concentration is increased, the double reciprocal plot becomes increasingly nonlinear at low NADH concentrations, but remains linear at high NADH levels. The apparent Kᵢ for NAD⁺ as determined by the method of Dixon (4) is 20 μM, in the case of the purified enzyme. The membrane-bound enzyme is inhibited by NAD⁺ with a Kᵢ of 70 μM (Table II).

The specificity for NADH as a substrate, and the inability to use NADPH, is mirrored in the response to pyridine nucleotide inhibitors. There is no inhibition of the enzyme by NADP⁺, NADPH, or NMN.

Double reciprocal plots of reaction velocity versus NADH concentration at different concentrations of AMP show that...
AMP, ATP, and ADP are competitive inhibitors. The apparent $K_i$ values for the three nucleotides were obtained from Dixon plots, and are listed in Table II for the purified enzyme, as well as for the membrane-bound enzyme and NADH oxidase. The apparent $K_i$ of the purified enzyme for ATP is 8 to 15 times greater than that for ADP and AMP, and the same relationship holds for the membrane-bound dehydrogenase and oxidase.

Other adenine nucleotides inhibit the enzyme, as shown in Table III, where their effects are contrasted to those of AMP, ADP, and ATP, at both high and low NADH concentrations. Adenine, deoxyadenosine, and deoxy-AMP, at the concentrations tested, were effective only at low NADH concentrations, indicating that they are relatively weak competitive inhibitors. CAMP is less effective than ADP at concentrations much above physiological levels of the nucleotide.

Many other compounds have been tested for their possible roles as effectors of the purified respiratory chain NADH dehydrogenase. The following compounds did not inhibit the enzyme at the concentrations shown: NADP, NMN, NADPH (all 0.5 mM); acetyl-CoA (1 mM); GTP, UTP, CTP, deoxy-CTP, deoxy-ATP, deoxy-GTP (all 3 mM); GMP, UMP, CMP, GDP, UDP, CDP (all 5 mM); deoxy-CMP, deoxycytidine, uridine, cytosine, cytidine, 5-methyl cytosine, thymine, acetate, pyruvate, glutamine, glutamate, lactate, malate, citrate, isocitrate, α-ketoglutarate, succinate, aspartate, glycine (all 10 mM).

Preparation and Characterization of Antiserum Directed Against the Respiratory Chain NADH Dehydrogenase—Antibodies to the NADH dehydrogenase were elicited with the nearly homogeneous enzyme preparation, estimated to be 75% pure. The results of immunoelectrophoresis using a somewhat less pure preparation of NADH dehydrogenase (about 30% pure, as estimated by sodium dodecyl sulfate gel electrophoresis) and of Triton-solubilized membrane vesicles, are shown in Fig. 6. The results with the purified enzyme show one major precipitin band, and two very minor bands that were barely visible (Fig. 6A). Only the major band stained for NADH dehydrogenase activity. Two major precipitin bands are seen following immunoelectrophoresis of Triton-solubilized membranes (Fig. 6B). Only the upper precipitin band stained enough to detect enzyme activity. However, the staining reaction was not intense enough to determine whether the second major band in Fig. 6B had any NADH dehydrogenase activity. Thus, aside from the fact that it reacts with the antiserum prepared from the purified NADH dehydrogenase, the relationship of the second band to the enzyme is not clear.

The results of Ouchterlony double diffusion analysis of the antiserum are shown in Fig. 7, for both crude, solubilized membrane vesicles, and a partially purified enzyme preparation. The results with the partially purified enzyme indicate that there are at least three immunologically distinct antigens present in the preparation (Fig. 7; antibody well A, antigen wells 1 to 3). With undiluted enzyme (antigen well 1) only two precipitin bands are distinguishable; dilution of the enzyme (antigen wells 2 and 3) resolves the band which was equidistant between the antibody and antigen wells into two distinct precipitin bands.

![Fig. 7. Immunodiffusion of partially purified and crude Triton-solubilized NADH dehydrogenase. Immunodiffusion was performed as described under “Materials and Methods” on plates containing 0.1% Triton X-100. Both antibody wells, marked as A and B, received 92 µg of crude, anti-NADH dehydrogenase γ-globulin. In A, antigen wells 1 to 3 received 5 µg, 2.5 µg, and 1.3 µg of untreated purified enzyme, respectively; antigen wells 4 to 6 received (respectively) 5 µg, 2.5 µg and 1.3 µg of purified enzyme treated with 0.1% sodium dodecyl sulfate. In B, antigen wells 1-3 received 25 µg (1), 12.5 µg (2), and 6.3 µg (3) of Triton-solubilized membrane vesicles. Antigen wells 4-6 received 25 µg (4), 12.5 µg (5), and 6.3 µg (6) of Triton-solubilized membrane vesicles, treated with 0.1% sodium dodecyl sulfate. Sodium dodecyl sulfate-treated samples were prepared by incubating purified enzyme (1 µg/ml) or Triton-solubilized membrane vesicles protein (5 µg/ml) for 30 min at 22° after adding sodium dodecyl sulfate to a final concentration of 0.1%. The partially purified enzyme used was estimated to be about 30% pure.](http://www.jbc.org/)
Treatment of the purified enzyme preparation with 0.1% sodium dodecyl sulfate (antigen wells 4, 5, and 6) diminishes the intensity of the precipitin band nearest the antigen well. It was previously shown in this laboratory and elsewhere \((1, 15)\) that low concentrations of sodium dodecyl sulfate do not inhibit many immunoprecipitation reactions. The pattern obtained with undiluted sodium dodecyl sulfate-treated samples (antigen well 4) resembles that obtained with the corresponding, untreated enzyme preparation (antigen well 1). If the preparation is diluted prior to treatment with sodium dodecyl sulfate (antigen wells 5 and 6), the precipitin band nearest to the antigen well disappears, while the two precipitin bands about equidistant from the antigen and antibody wells appear unaltered. Although not clearly shown in Fig. 8A, further dilution of the antiserum revealed, with sodium dodecyl sulfate-treated antigen preparations, a weak precipitin band near the antibody well that is not found in untreated samples.

As shown in Fig. 7A, the double diffusion technique gives a more complex pattern of immunoprecipitants than was obtained by immunoelectrophoresis. The reason for this difference is not known. We have not been able to determine which of the precipitin bands corresponds to the enzyme, since the stain for enzyme activity was not intense enough to be seen. However, the results of immunoelectrophoresis (Fig. 6A) indicate that the enzyme is the major antigen present in purified enzyme samples.

The results of immunodiffusion of the \(\gamma\)-globulin fraction against the Triton-solubilized membranes (Fig. 7B) are somewhat different from those obtained in the case of the partially purified enzyme (Fig. 7A). With Triton-solubilized membranes a large amount of material does not leave the antigen well during the double diffusion analysis (Fig. 7B; wells 1, 2, and 3). Since certain antigens might not be detected because of their being trapped in the antigen wells, the antigens were treated with sodium dodecyl sulfate in an attempt to disaggregate them further. Sodium dodecyl sulfate treatment almost entirely eliminates the problem of antigen trapping in the wells (compare Fig. 7B, wells 1 to 3 with 4 to 6). Several minor precipitin bands were found after sodium dodecyl sulfate treatment of the antigen, but these are not seen very well in the photograph.

The position of the major precipitin band was unchanged by sodium dodecyl sulfate treatment. When purified enzyme was eluted from sodium dodecyl sulfate-polyacrylamide gels after electrophoresis, only one precipitin band was obtained (data not shown). Thus, the major band seen on gels of purified enzyme appears to be antigenically homogeneous. The other minor precipitin bands obtained with the less purified preparation of enzyme used in Fig. 6A and 7A arise from minor contaminants present in these preparations.

The immunoelectrophoresis and double immunodiffusion data of Figs. 6 and 7 show that the antisera are directed against one principal antigen of the purified NADH dehydrogenase, and that this antigen is the enzyme. The antisera react with one major component in unfractionated solubilized membranes (Fig. 6B). The possible relationship of this other component to the enzyme is discussed below (see “Discussion”).

Inhibition of NADH Dehydrogenase by Antiserum—The antisera preparation characterized above was reacted with both purified enzyme and membrane-bound NADH dehydrogenase, in order to examine the effect of antiserum on enzyme activity. As shown in Fig. 8A, the anti-NADH dehydrogenase \(\gamma\)-globulin fraction inhibits NADH dehydrogenase in crude enzyme preparations. Saturating concentrations cause a maximum inhibition of about 60%. Longer incubation did not result in any further decrease in activity relative to the control, which was incubated with a nonimmune \(\gamma\)-globulin fraction. The experiment of Fig. 8A was done with a crude preparation of the enzyme, prepared as described in the legend. It has been difficult to obtain similar data for the purified enzyme because it is labile to prolonged incubation above 0° in the absence of antibody in the reaction mixture. Enzyme (10 \(\mu\)g) and the indicated amount of crude \(\gamma\)-globulin from either preimmune (●●●●), or anti-NADH dehydrogenase (○○○○) serum were preincubated in the assay mixture for 1 min prior to the addition of NADH, to start the reaction. C, inhibition of NADH oxidase and membrane-bound NADH dehydrogenase by anti-NADH dehydrogenase antibodies. Incubation mixtures contained, in a final volume of 900 \(\mu\)l: 300 \(\mu\)g of membrane protein, 10 mM potassium phosphate (pH 7.1), 0.15 M NaCl, and the indicated amount of either preimmune control \(\gamma\)-globulin (●●●●) or anti-NADH dehydrogenase (○○○○) crude \(\gamma\)-globulin. Samples were incubated for 1 h at 22° and then 3 hr at 0-4°. Aliquots were removed for assay of NADH oxidase (●●●●) or NADH dehydrogenase (○○○○) as described under “Materials and Methods.”
subunits, seen even in the uninhibited reaction when menadione is the electron acceptor (Fig. 3), but which is emphasized in the presence of the inhibitor, NAD', being essentially the same for the 2 forms. Thus, in contrast to the mitochondrial enzyme, the isolated E. coli enzyme has properties very much like those of the membrane-bound enzyme. Nonetheless, there is some evidence that the enzyme in situ may be associated with other membrane components (see below).

Because of the central position of NADH in metabolism, we were interested in discovering whether the NADH dehydrogenase was subject to feedback inhibition by critical intermediary metabolites. As shown in Tables II and III, adenine nucleotides inhibit the enzyme in a rather nonspecific fashion. In the cases where this inhibition was examined in more detail, it was found to be competitive, even in the case of adenine itself (Table III). The most effective inhibitor found for the enzyme is NAD', the product of the reaction. The next most effective inhibitor is AMP (Table II), with ADP being only slightly less effective, and ATP substantially less so. The most reasonably explanation for the inhibitory effects of adenine compounds seems to be that the adenine nucleotides mimic the effects of NAD' as an inhibitor of the enzyme, perhaps by occupying an adenine-specific site. This data is substantiated by the finding that NMN, lacking the adenine moiety, is not inhibitory. Many other NADH-dependent dehydrogenases have a specific adenine binding site at the pocket for cofactor interaction with the enzyme (18). Thus, we surmise that the inhibitory effects of other adenine nucleotides are based on their similarity to the true inhibitor, NAD'. We have examined a number of other nucleotides and intermediary metabolites for their inhibitory effects and have seen no inhibition or stimulation of the purified enzyme. In many of these cases, inhibition was tested on the membrane-bound enzyme with, again, no effect. Thus, the principal regulation of the enzyme at the level of feedback inhibition seems to occur in the interaction of NADH and NAD' with the enzyme. The $K_m$ for NAD' is about the same as the apparent $K_m$ for NADH (Tables I and II). Since the ratio of NAD' to NADH in aerobically growing E. coli cells is around 3.5 (19), the enzyme should always be partially inhibited and

**Table IV**

<table>
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<tr>
<th>Addition</th>
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</tr>
<tr>
<td>Control γ-globulin</td>
<td>1</td>
</tr>
<tr>
<td>Anti-NADH dehydrogenase γ-globulin</td>
<td>46</td>
</tr>
</tbody>
</table>

The purified enzyme is stabilized against this loss of activity by antibodies (Table IV). If the purified enzyme is incubated with anti-NADH dehydrogenase γ-globulin under conditions which result in almost complete loss of activity in controls, about 46% of the initial activity can be recovered. The crude enzyme retains approximately the same amount of activity in the presence of enzyme (Fig. 5A).

In order to avoid prolonged preincubation of purified enzyme with antiserum, with attendant loss of activity of uncomplexed enzyme, the antibody fraction was added directly to the assay mixture. As shown in Fig. 5B, the enzyme is inhibited by about 50% with saturating amounts of immune γ-globulin. In addition, there is more inhibition by immune serum than was seen with the crude enzyme. To explore further the relationship between the purified enzyme, the membrane-bound NADH dehydrogenase, and the NADH oxidase, the latter activities were measured after preincubation of membrane vesicles with control and immune γ-globulin (Fig. 5C). Both the NADH dehydrogenase and NADH oxidase were inhibited by 50 to 60%, which is the same as the maximum inhibition obtained with the solubilized enzyme.

**DISCUSSION**

The purified respiratory chain NADH dehydrogenase can use several different electron acceptors (Table I), however, it has specificity in its electron donor, in that it oxidizes NADH, use several different electron acceptors (Table I), however, it has specificity in its electron donor, in that it oxidizes NADH, and ATP substantially less so. The most reasonably explanation for the inhibitory effects of adenine compounds seems to be that the adenine nucleotides mimic the effects of NAD' as an inhibitor of the enzyme, perhaps by occupying an adenine-specific site. This data is substantiated by the finding that NMN, lacking the adenine moiety, is not inhibitory. Many other NADH-dependent dehydrogenases have a specific adenine binding site at the pocket for cofactor interaction with the enzyme (18). Thus, we surmise that the inhibitory effects of other adenine nucleotides are based on their similarity to the true inhibitor, NAD'. We have examined a number of other nucleotides and intermediary metabolites for their inhibitory effects and have seen no inhibition or stimulation of the purified enzyme. In many of these cases, inhibition was tested on the membrane-bound enzyme with, again, no effect. Thus, the principal regulation of the enzyme at the level of feedback inhibition seems to occur in the interaction of NADH and NAD' with the enzyme. The $K_m$ for NAD' is about the same as the apparent $K_m$ for NADH (Tables I and II). Since the ratio of NAD' to NADH in aerobically growing E. coli cells is around 3.5 (19), the enzyme should always be partially inhibited and

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*G. F. Dancey and B. M. Shapiro, manuscript in preparation*
should respond cooperatively to increases in NADH concentra-
tion (Fig. 5). Thus, in the absence of further information about the 
metabolic regulation of this enzyme, it appears that feedback regulation at the level of enzyme activity might occur as a secondary effect, in response to alterations of NAD\(^+\) and 
NADH levels by controls in the other metabolic pathways.

The antisera against NADH dehydrogenase have further 
clarified the role of the enzyme in the respiratory chain. 
Analysis of the antisera by both immunoelectrophoresis and 
Ouchterlony double diffusion (Figs. 6 and 7) show that there is quite specific reaction with the purified enzyme. However, 
when solubilized membranes are examined, the reactivity of the 
antiserum is much more complex. This is especially 
pronounced when crossed immunoelectrophoresis (Fig. 6), 
where a second major immunoprecipitating component is 
found in Triton-solubilized membranes. The staining of 
immunoprecipitates was sensitive enough to barely detect the 
enzyme activity in the major precipitin band. Because of the 
faintness of the staining, we were unable to detect activity (or 
equivalently demonstrate its absence) in the second band. 
However, the existence of a second cross-reactive band, when 
considered together with the 2 bands of NADH-DCIP reduc-
tase seen when Triton-solubilized membranes are separated by 
gel electrophoresis (Fig. 1 of Ref. 1), and other results which 
suggest that the second band seen in Triton-solubilized mem-
branes contains as one of its components the 38,000-dalton 
NADH dehydrogenase band,\(^3\) implies that the NADH dehy-
drogenase exists in the membrane associated with other 
components still to be identified. Thus, we are exploring the 
possibility that the 38,000-dalton component is the active subunit of an NADH dehydrogenase complex, which may have 
other components of the electron transport chain closely 
associated with it.

The antibodies directed against the respiratory chain NADH 
dehydrogenase inhibit its activity by about 50%. This inhibition 
tends whether the enzyme is present in solubilized 
membrane preparations or in a purified form, however, in the 
latter case the enzyme is unstable and must be assayed directly in 
the reaction mixture in order to preserve activity (Fig. 8C). 

The enzyme of crude membrane preparations is stable to 
prolonged incubation and thus may be more readily examined. 
In all cases the inhibition by antibody is approximately 50%. 
Although the enzyme is labile to prolonged incubation, it may 
be stabilized by the antibody preparation (Table IV). Since the 
amount of activity seen with maximum stabilization is around 
50%, and that is the extent of inhibition by the antibody, we 
interpret the results in Table IV to mean that the enzyme is 
completely stabilized and 50% inhibited by the antiserum

\(^3\) G. F. Dancey, unpublished data.

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