STUDIES OF A HALOPHILIC NADH DEHYDROGENASE

I. PURIFICATION AND PROPERTIES OF THE ENZYME

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SUMMARY

1. An NADH dehydrogenase obtained from an extremely halophilic bacterium was purified 570-fold by a combination of gel filtration, chromatography on hydroxyapatite, and ion-exchange chromatography on QAE-Sephadex.

2. The purified enzyme appeared to be FAD-linked and had an apparent molecular weight of 64,000.

3. Even though enzyme activity was stimulated by NaCl, considerable activity (43% of the maximum activity observed in the presence of 2.5 M NaCl) was observed in the absence of added NaCl.

4. The enzyme was unstable when incubated in solutions of low ionic strength. The presence of NADH enhanced the stability of the enzyme.

INTRODUCTION

We previously described the oxidation of NADH by crude extracts prepared from an extremely halophilic bacterium (designated as strain AR-I) and showed that high concentrations of NaCl were required for maximum activity of the enzyme. These studies suggested that the salt requirement could not be fully explained in terms of charge neutralization since, in addition to certain anomalous cation effects, anions were found to markedly affect the ability of monovalent cations to activate the enzyme. Furthermore, the requirement for NaCl could be satisfied by mM concentrations of Mg²⁺ salts or μM concentrations of polyamines such as spermine. These observations were subsequently confirmed by others and it was proposed that the requirement for high concentrations of NaCl, in the case of a membrane-bound menadione reductase obtained from Halobacterium cutirubrum, reflected the ability of certain ions to stabilize hydrophobic interactions.

Abbreviations: NADH dehydrogenase, reduced NAD: 2,6-dichlorophenolindophenol oxidoreductase; DCIP, 2,6-dichlorophenolindophenol; TES, N-tris-(hydroxymethyl)-methyl-2-amino-ethanesulfonic acid; QAE, diethyl-(2-hydroxypropyl)aminoethyl.
In order to study these various ionic effects in a system uncomplicated by the presence of a multi-enzyme system, particularly one enriched with respect to the hydrophobic components associated with bacterial membranes, an attempt was made to separate and purify the reduced-NAD:2,6-dichlorophenolindophenol oxidoreductase, EC 1.6.99.3 (NADH dehydrogenase) component from the electron-transport chain of strain AR-I.

Although enzymes from halophilic bacteria are extremely labile in solutions of low ionic strength, certain observations suggested that several techniques used to purify nonhalophilic enzymes might be applicable to purification of the NADH dehydrogenase from AR-I. For example, the bulk proteins from several extremely halophilic bacteria contain large amounts of glutamic and aspartic acids resulting in relatively acidic proteins. On the assumption that the NADH dehydrogenase from AR-I was likewise acidic, chromatography on hydroxyapatite seemed potentially useful since carboxyl groups were reported responsible for protein–hydroxyapatite interaction. In addition, polyamines, such as spermine, stabilized NADH dehydrogenase activity in solutions of low ionic strength suggesting that ion-exchange chromatography, if carried out in the presence of a suitable stabilizing agent, could be used to purify the enzyme. Subsequently, NADH was found to be a more effective stabilizing agent than spermine. Finally, the NADH dehydrogenase from AR-I, like certain other enzymes obtained from extremely halophilic bacteria, was reactivated by the addition of optimal concentrations of NaCl following inactivation by exposure to solutions of low ionic strength.

The present paper describes some properties of the enzyme following purification by agarose-gel filtration, hydroxyapatite chromatography, and ion-exchange chromatography on diethyl-(2-hydroxypropyl)aminoethyl-Sephadex (QAE-Sephadex). The final product, recovered in about 30% yield, catalyzed the oxidation of 376 μmoles of NADH per min per mg protein.

**MATERIALS AND METHODS**

**Standard assay**

NADH dehydrogenase activity was determined as previously described except that bovine serum albumin was added to a final concentration of 40 μg/ml to stabilize the enzyme during the assay. While the rate of dye reduction was proportional to the amount of added enzyme, these conditions did not measure the total amount of NADH dehydrogenase activity since the concentration of 2,6-dichlorophenolindophenol (DCIP) employed was only 2.3 times greater than its apparent K_m. Higher concentrations of dye were inhibitory. A unit of activity is that amount of enzyme that reduces 1 μmole of DCIP per min using standard assay conditions. Specific activity is defined as units/mg protein. The millimolar absorption for DCIP (20 mM−1·cm−1) was unaffected by the NaCl concentration.

**Protein determinations**

The protein concentration was determined either from the absorbance at 280 nm or by the Waddell procedure as described by Murphy and Kies. The presence of NADH in the QAE fraction interfered with the Waddell procedure. Therefore, NADH was removed by passing an appropriate aliquot of the enzyme through a
0.7 cm × 20 cm volume of G-50 Sephadex equilibrated with 10 mM NaCl. The volume of effluent corresponding to the volume in which the blue dextran was eluted was employed for the protein determination. In control experiments using bovine serum albumin, from 98–103% of the added protein was recovered.

**Reactivation**

The enzyme, inactivated by incubating it in solutions of low ionic strength, was reactivated by the addition of solid NaCl to the enzyme to a final concentration of 3 M NaCl. Incubation at room temperature (approx. 22 °C) was the most effective temperature for reactivation, which was usually complete after 24–36 h.

**Concentration of the enzyme**

The volume of enzyme was reduced by ultrafiltration in an Amicon UF cell equipped with a PM-10 membrane. The cell was placed in an ice bath and operated at a pressure of 75 lb/inch² with nitrogen.

**Chemicals and reagents**

Hydroxyapatite (Bio-Gel HT), agarose gel (Bio-Gel A-0.5 m), and polyacrylamide gel (Bio-Gel P-10 and P-200) were products of Bio-Rad Laboratories. QAE-Sephadex (A-50), various Sephadex gels, and Blue Dextran were obtained from Pharmacia Fine Chemicals. All the gels and the QAE-Sephadex were swollen for 72 h at room temperature in appropriate buffers. Bio-Gel HT was suspended in 1.0 M NaCl-100 mM Tris–HCl-100 μM NADH (pH 7.4), allowed to settle several times (decanting the fines each time), and equilibrated with at least three column volumes of the above buffer before use.

Bovine serum albumin (Fraction V), DCIP, and NADH were obtained from the Sigma Chemical Company. Deamino-NADH and the NAD analogs were products of P.L. Biochemicals, Inc. The oxidized analogs were reduced with horse liver alcohol dehydrogenase obtained from Boehringer. The products were recovered by passing the protein-free supernatant (prepared by placing reaction mixtures in a boiling water bath for 2 min and centrifuging for 10 min at 10,000 × g) through a 1.5 cm × 25 cm column of G-10 Sephadex which was equilibrated with 100 mM NaCl. The analogs, located spectrophotometrically, were well separated from the other components of the reaction mixture. The peak fractions were combined and concentrated by lyophilization. The absorbance of the cyanide complex of the oxidized analogs and the absorbance of the reduced analogs were used to determine their concentrations. The spectral data and constants required for these determinations were obtained from P.L. Biochemicals, Inc. Reduction of the analogs was quantitative and the overall recoveries ranged from 50–94% of theory.

Molecular weight determinations were carried out according to the method of Andrews using a 1.5 cm × 24.5 cm column of P-200 polyacrylamide gel previously equilibrated with 2 M NaCl-100 mM Tris–HCl (pH 7.4). The column was calibrated with lactic dehydrogenase, fumarase, and lysozyme, obtained from Boehringer, and bovine serum albumin.

All other chemicals were obtained from the usual commercial sources.

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**HALOPHILIC ENZYMES**

**RESULTS**

**Gel filtration**

80 mM NaCl was removed from the enzyme as the dilution was made to NaCl-100 mM Tris–HCl (pH 7.4) which prevented NADH dehydrogenase activity. The protein was eluted from the gel column, and the absorbance at 280 nm was recorded.

**Fig. 1.** Agarose gel protein and DNA profile. The gel previously used for purification was cut into 2 cm sections and the absorbance at 280 nm was recorded from 65–95% of the gel.

When the gel was run in 1 M HCl (pH 7), no protein appeared indicating the protein was eluted from the remaining gel matrix of the column.

**Hydroxyapatite**

22.5 cm × 20 cm Bio-Gel HT-100 gel was equilibrated with 1.0 M NaCl-100 mM Tris–HCl (pH 7.4). The column was calibrated with lactic dehydrogenase, fumarase, and lysozyme, obtained from Boehringer, and bovine serum albumin.

All other chemicals were obtained from the usual commercial sources.
RESULTS

Gel filtration

80 ml of crude extract (prepared as previously described) were diluted to a concentration of 22.9 mg protein per ml using 2 M NaCl–100 mM Tris–HCl (pH 7.4) as the diluent and passed through an agarose column equilibrated with 2 M NaCl–100 mM Tris–HCl (pH 7.4). As shown in Fig. 1, NADH dehydrogenase activity appeared at a $V_e/V_o$ of 1.5 and was separated from carotenoid-containing fractions which presumably were associated with membrane-rich materials. 1330 units of NADH dehydrogenase (spec. act. 0.72), were applied to the column. Following gel filtration, combination and concentration of Fractions 49–55 yielded a total of 1290 enzyme units at a specific activity of 6.4. Recoveries were usually 75% but ranged from 65–97%. The agarose fraction could be stored at $-120 \, ^\circ C$ for at least 3 months with little loss of activity.

When gel filtration was carried out in the presence of 4 M NaCl–100 mM Tris–HCl (pH 7.4), 77% of the NADH dehydrogenase activity present in the crude extract appeared coincident with those fractions containing the carotenoid pigment. The remaining NADH dehydrogenase activity appeared in the included volume of the column.

Hydroxyapatite chromatography

22.5 ml of the agarose fraction were placed on a 2.5 cm $\times$ 29.5 cm column of Bio-Gel HT previously equilibrated with 1 M NaCl–100 $\mu$M NADH–100 mM Tris–HCl (pH 7.4).

Following adsorption of the agarose fraction, the gel was washed with 185 ml of 1.0 M NaCl–100 $\mu$M NADH–100 mM Tris–HCl (pH 7.4). This resulted in the elution of some protein, but no NADH dehydrogenase (Fig. 2). The column was
Fig. 2. Hydroxyapatite chromatography of the agarose fraction. 22.5 ml of the agarose fraction, containing 8.75 mg of protein per ml and a total of 1265 units, were placed on a 2.5 cm × 29.5 cm column of Bio-Gel HT equilibrated in 1.0 M NaCl-100 μM NADH-100 mM Tris-HCl (pH 7.4). The column was washed with 185 ml of the above buffer. Subsequently, the column was washed sequentially as indicated with ammonium phosphate-1.0 M NaCl-100 μM NADH buffers (pH 8.5) containing the following concentrations of ammonium phosphate: 150, 250, and finally 500 mM. 12-ml fractions were collected at a flow rate of 48 ml/h. The column was operated at room temperature (approx. 22 °C). NADH dehydrogenase (○—○); A₂₈₀ nm (●—●).

washed sequentially with three column volumes of ammonium phosphate buffers (at pH 8.5) at the following concentrations: 150, 250, and 500 mM. Each of the buffers were 1.0 M with respect to NaCl and 100 μM with respect to NADH. As shown in Fig. 2, a large amount of protein but no enzyme activity was eluted with 150 mM ammonium phosphate. When the wash was changed to 250 mM ammonium phosphate, some protein was eluted followed by the enzyme. Additional protein but no NADH dehydrogenase activity was eluted with 500 mM ammonium phosphate. Fractions 68–79 were combined, concentrated, and designated as the HA fraction. About 60% of the units applied to the column were recovered with a 25-fold increase in spec. act.

QAE-Sephadex fractionation

Five HA fractions were combined, concentrated, and passed through a 1.5 cm × 31 cm G-25 Sephadex column equilibrated, in 350 mM NaCl-100 μM NADH-10 mM Na TES (pH 7.0). The NADH dehydrogenase was excluded from the column and was subsequently placed on a 2.5 cm × 16 cm QAE-Sephadex column previously equilibrated with 350 mM NaCl-100 μM NADH-10 mM Na TES (pH 7.0). After the addition of enzyme, the column was washed with 350 ml of the starting buffer. Thereafter, an NaCl gradient was established from 350 to 500 mM NaCl over a total volume of 400 ml. The enzyme was eluted from the column between 420 and 450 mM NaCl (Fig. 3). Those fractions of highest specific activity (Fractions 61–79) were combined and immediately reactivated. Following reactivation, the enzyme (QAE fraction) was concentrated, made 0.8% with respect to bovine serum albumin, and stored at −120 °C.

Table I summarizes the purification procedure. The QAE fraction oxidized 376 μmoles of NADH per min per mg protein which represented a 545-fold puri-
HALOPHILIC NADH DEHYDROGENASE: PROPERTIES

Fig. 3. QAE-Sephadex chromatography of fraction HA. 21 ml of fraction HT (containing 26.8 mg protein per ml and a total of 3420 units of enzyme) were passed through a 1.5 cm x 31 cm G-25 Sephadex column equilibrated in 350 mM NaCl-100 mM NADH-10 mM Na TES (pH 7.0). The enzyme was then placed on a 2.5 cm x 16 cm column of QAE-Sephadex (A-50 previously equilibrated in 350 mM NaCl-100 mM NADH-10 mM Na TES (pH 7.0). Following absorption of the enzyme to the column, the column was washed with 350 ml of the same buffer until the absorption at 280 nm reached a low and relatively constant value. Thereupon, a linear NaCl gradient was established in 10 mM Na TES-100 mM NADH (pH 7.0) from 350 to 500 mM NaCl over a total volume of 400 ml. 5 ml fractions were collected at a flow rate of 36 ml/h. The column was operated at 4 °C. NADH dehydrogenase activity (O--O); A280 nm (●—●).

fication of the activity present in the crude extract. The QAE fraction was colorless and could be stored at −120 °C for at least 1 year without any loss of activity. When supplemented with bovine serum albumin to a final concentration of 0.8%, the enzyme could be frozen and thawed repeatedly with little loss of activity. Samples have been stored 4 °C for 1 month with less than a 10% loss of the initial activity.

Properties of the enzyme

While NADH dehydrogenase activity was stimulated by the presence of NaCl (Fig. 4), considerable activity was observed in the absence of added salt. Extrapolation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total volume (ml)</th>
<th>Total units</th>
<th>Recovery (%)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Purified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>400</td>
<td>6300</td>
<td>100</td>
<td>9130</td>
<td>0.69</td>
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<td>125</td>
<td>5940</td>
<td>94</td>
<td>1110</td>
<td>5.35</td>
<td>7.8</td>
</tr>
<tr>
<td>HA</td>
<td>21</td>
<td>3420</td>
<td>54</td>
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<td>128</td>
<td>186</td>
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<tr>
<td>QAE</td>
<td>136</td>
<td>1880</td>
<td>30</td>
<td>5.0</td>
<td>376</td>
<td>545</td>
</tr>
</tbody>
</table>
Fig. 4. Effect of NaCl on NADH dehydrogenase activity. Enzyme activity was determined in standard reaction mixtures in which only the NaCl concentration was varied as indicated. 0.03 unit of QAE fraction were assayed at each concentration of salt. When assayed in the absence of added NaCl, the concentration of NaCl was 15 mM, due to carry over of NaCl with the enzyme.

Fig. 5. Effect of DCIP on NADH dehydrogenase activity. NADH dehydrogenase activity was determined in standard reaction mixtures in which only the DCIP concentration was varied as indicated. 0.03 unit of QAE fraction were assayed at each concentration and the average of three determinations at each concentration are plotted.

to zero NaCl concentration gave a rate of NADH oxidation some 43% of the maximum rate observed at 2.5 M NaCl. The apparent $K_m$ for DCIP, in the presence of 100 $\mu$M NADH, was 30 $\mu$M (Fig. 5). The enzyme was inhibited by DCIP at concentrations greater than 70 $\mu$M. The apparent $K_m$ for NADH, in the presence of 70 $\mu$M DCIP, was 23 $\mu$M. No inhibition of dye reduction was observed at concentrations of NADH as high as 400 $\mu$M (Fig. 6). The maximal velocity when extrapolated to a saturating concentration of NADH (385 $\mu$moles/min per mg protein) was about 70% of the extrapolated maximal velocity at a saturating concentration of DCIP.

Fig. 6. Effect of NADH on NADH dehydrogenase activity. NADH dehydrogenase activity was determined in standard reaction mixtures in which only the NADH concentration was varied, as indicated. 0.03 unit of QAE fraction were assayed at each concentration and the average of three determinations is plotted.

TABLE II

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCIP</td>
<td></td>
</tr>
<tr>
<td>Menadione</td>
<td></td>
</tr>
<tr>
<td>Juglone</td>
<td></td>
</tr>
</tbody>
</table>

two were only slightly affected by the presence of vitamin K$_1$ (1-naphthol) or cytochrome c.

Specificity of NADH

The specificity of NADH for NADH dehydrogenase was determined by the rates of dye reduction at saturating concentrations of non-enzymic acceptors, summarized in Table II. The nature of the oxidant affected the specificity of NADH; the enzyme was inhibited by DCIP at concentrations greater than 70 $\mu$M. The apparent $K_m$ for NADH, in the presence of 70 $\mu$M DCIP, was 23 $\mu$M. No inhibition of dye reduction was observed at concentrations of NADH as high as 400 $\mu$M (Fig. 6). The maximal velocity when extrapolated to a saturating concentration of NADH (385 $\mu$moles/min per mg protein) was about 70% of the extrapolated maximal velocity at a saturating concentration of DCIP.

No variation in the presence was constant. The Tris-acetate, pH 7.5, at these acceptor of non-enzymic

Specificity

A limitation to those tested was (5-hydroxy

<table>
<thead>
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<th>Electron acceptor</th>
<th>Specificity</th>
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</thead>
<tbody>
<tr>
<td>DCIP</td>
<td></td>
</tr>
<tr>
<td>Menadione</td>
<td></td>
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<tr>
<td>Juglone</td>
<td></td>
</tr>
</tbody>
</table>

two were only slightly affected by the presence of vitamin K$_1$ (1-naphthol) or cytochrome c.
No well defined pH optimum was observed when the enzyme was assayed in the presence of 2.5 M NaCl-50 mM imidazole-HCl buffer. The rate of dye reduction was constant from pH 6.6 to pH 7.0. At pH values greater than 7, the rate of dye reduction rapidly decreased. When assayed at pH values more acidic than 6.6 (using Tris-acetate buffers) the rate of dye reduction apparently increased. However, assays at these acidic pH values were complicated by the presence of an extremely high rate of non-enzymic dye reduction and made the acquisition of reliable data difficult.

Specificity of electron acceptors

A limited number of oxidants served as electron acceptors for the enzyme. Of those tested, only DCIP, menadione (2-methyl-1,4-naphthoquinone), and juglone (5-hydroxy-1,4-naphthoquinone) were reduced by the enzyme (Table II). The latter two were only 60% as effective as DCIP when measured at saturating concentrations of oxidant. The maximal velocity, at a saturating concentration of NADH, was also affected by the nature of the oxidant with the greatest activity observed in the presence of DCIP. The apparent $K_m$ for NADH was relatively unaffected by the nature of the electron acceptor.

The following electron acceptors were not reduced by the enzyme in the presence of NADH: potassium ferricyanide, lawson (2-hydroxy-1,4-naphthoquinone) vitamin $K_1$ (2-methyl-3-phytyl-1,4-naphthoquinone), vitamin $K_5$ (4-amino-2-methyl-1-naphthol), coenzyme $Q_6$, coenzyme $Q_{10}$, lipoic acid, lipoamide, and horse heart cytochrome $c$.

Specificity of electron donor

The substrate specificity of the enzyme was investigated at various concentrations of reduced NAD analogs and DCIP. The results of these experiments are summarized in Table III. Of the analogs tested, only 3-acetylpyridine-NADH, 3-acetylpyridine-deamino-NADH, and thionicotinamide-NADH were oxidized. The rates of dye reduction, extrapolated to infinite substrate concentration, varied from 35-47% of the rate observed at saturating concentrations of NADH. NADPH,
TABLE III

**SUBSTRATE SPECIFICITY OF THE NADH DEHYDROGENASE**

The QAE-fraction was assayed in the presence of variable concentrations of oxidant and substrate. When the oxidant concentration was varied, the following substrate concentrations were employed: 3-acetylpyridine deamino-NADH, 800 μM; thionicotinamide-NADH, 570 μM; 3-acetylpyridine NADH, 630 μM. The DCIP concentration was 70 μM when the substrate concentration was varied. The reported values for V and K_m were obtained from double reciprocal plots. V is reported as μmoles of DCIP reduced per min per mg protein at saturating concentrations of oxidant or NADH.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate</th>
<th>DCIP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V (μM)</td>
<td>V (μM)</td>
</tr>
<tr>
<td>NADH</td>
<td>385</td>
<td>568</td>
</tr>
<tr>
<td>3-Acetylpyridine deamino-NADH</td>
<td>180</td>
<td>146</td>
</tr>
<tr>
<td>Thionicotinamide-NADH</td>
<td>173</td>
<td>241</td>
</tr>
<tr>
<td>3-Acetylpyridine-NADH</td>
<td>135</td>
<td>227</td>
</tr>
</tbody>
</table>

deamino-NADH, 3-pyridine aldehyde NADH, and 3-pyridine aldehyde deamino-NADH did not serve as substrates.

When NADH oxidation was carried out in the presence of the various inactive analogs, only 3-pyridine aldehyde NADH inhibited substrate oxidation. As shown in Fig. 7a, the inhibition was competitive with respect to NADH; the K_i was 400 μM, about 20 times greater than K_m for NADH. The patterns of substrate specificity and inhibition suggested that the presence of the 6-amino nitrogen in the purine ring was essential for substrate binding and that NAD was essential for the adenine ring of the adenylic acid.

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**Molecular weight**

Two different enzymes were assayed for the molecular weight using Sephadex G-200 in 2 M NaCl. When the enzyme was assayed for the molecular weight, 2 M NaCl was included in the column, NADH was not added to the substrate mixture.

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
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</tr>
<tr>
<td>FMN</td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 7. Inhibition of NADH dehydrogenase activity. 0.03 unit of QAE fraction were assayed in standard reaction mixtures containing the indicated concentration of NADH and inhibitor. The results are presented as the average of two determinations. (a) Inhibition by 3-pyridine aldehyde NADH; (b) inhibition by NAD; (c) inhibition by AMP; (d) effect of NMN on NADH dehydrogenase activity. Figures 7 (a-c) are Dixon plots whereas Fig. 7d is a double-reciprocal plot (O, 0.1 mM NMN; ●, no added NMN).
Fig. 8. Molecular weight of NADH dehydrogenase by gel filtration. 0.5 ml QAE fraction, containing 35 μg protein per ml, were placed on a 1.5 cm x 24.5 cm column of P-200 polyacrylamide gel previously equilibrated in 2 M NaCl-100 mM Tris-HCl (pH 7.4). The column was operated at room temperature (approx. 22 °C) and 1.0-ml fractions were collected. Fumarase (△), lactate dehydrogenase (□), bovine serum albumin (●), QAE fraction (○) and lysozyme (■) were used as markers.

Molecular weight determination

Two molecular weight determinations were made using P-200 gel (Fig. 8). The enzyme was eluted after bovine serum albumin monomer. The two values obtained for the molecular weight were 62,000 and 68,000. When a crude extract was diluted in 2 M NaCl-100 μM NADH-10 mM Na TES (pH 7.0) and passed through the P-200 column, NADH dehydrogenase activity was located at $V_E/V_0$ of 2.09 corresponding to the peak.

### TABLE IV

**Flavin content of the QAE-fraction**

0.5 ml of the QAE-fraction was analyzed for protein by following trichloroacetic acid extraction and hydrolysis. Flavin was calculated as the difference between the total flavin as determined by acid hydrolysis and the flavin present prior to hydrolysis. The latter was assumed to represent FMN.

<table>
<thead>
<tr>
<th>Component</th>
<th>Total content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg</td>
</tr>
<tr>
<td>Protein</td>
<td>19.1</td>
</tr>
<tr>
<td>FMN</td>
<td>0.029</td>
</tr>
<tr>
<td>FAD</td>
<td>0.296</td>
</tr>
</tbody>
</table>
to a molecular weight of 60,000. This suggested that following gel filtration subsequent purification did not produce an enzyme species of lower molecular weight.

**Flavin analysis**

Flavin analysis of the QAE fraction according to the method of Burch (Table IV) indicated that FAD represented 85% of the total flavin. The remaining flavin was presumably FMN. Although the presence of both flavins made identification of the flavin associated with the enzyme equivocal, the flavin to protein ratio was consistent with the assumption that FAD was associated with the enzyme. Assuming a flavin to protein ratio of 1, the minimum molecular weight for the enzyme was calculated to be 50,700 if FAD were the coenzyme (and 299,000 if FMN were the flavin). The former value was consistent with the molecular weight determined from polyacrylamide gel filtration (Fig. 8).

**Stability**

As shown in Fig. 9a, the enzyme was unstable in 100 mM and 250 mM NaCl when the salt concentration was adjusted by diluting the enzyme with buffer. In 100 mM NaCl, the loss of enzyme activity was immediate and exponential, whereas in 250 mM NaCl, the enzyme appeared stable for about 30 min before the loss of

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**DISCUSS**

Halophilic activity was stable during 4°C adjustment of the enzyme followed by filtration. The halophilic enzyme was concentrated in the first phase, 100 mM NaCl, has been reported to be stable at 4°C. Halobacterium halophilum has been reported to show halophilic activity in the same concentration of NaCl. The enzyme was stable in 4°C, with less than 25% of the total activity being reduced to disso...
activity became exponential. When incubated in 500 mM NaCl, the enzyme was stable during the time course of the experiment (150 min).

An entirely different response was observed when the salt concentration was adjusted by passing the enzyme through a P-10 polyacrylamide-gel column. The enzyme lost activity in a biphasic manner (Fig. 9b) in which a rapid initial phase was followed by a slower second one. In addition, there was no evidence of a "lag" prior to the loss of enzyme activity. When the enzyme was adjusted to a particular salt concentration by gel filtration using a buffer containing NADH, the loss of activity was considerably retarded (Fig. 9b). The first order decay constants for the second phase, in the absence of NADH, were calculated to be 0.035 min⁻¹ and 0.011 min⁻¹ in 100 and 250 mM NaCl, respectively. In the presence of NADH, they were 0.0026 min⁻¹ and 0.0013 min⁻¹.

**DISCUSSION**

The NADH dehydrogenase obtained from the halophilic bacterium AR-I exhibited two properties usually associated with enzymes obtained from extremely halophilic bacteria: instability in solutions of low ionic strength; stimulation of activity by high concentrations of NaCl. The stability of the NADH dehydrogenase from AR-I was markedly affected by the presence of its substrate (NADH). Similar substrate stabilization has been noted with other enzymes obtained from extremely halophilic bacteria. When the salt concentration was adjusted by dilution, the enzyme was more stable than when the salt concentration was adjusted by gel filtration. Furthermore, dilution also resulted in a lag prior to the loss of activity. These results are consistent with the hypothesis that dilution did not adequately reduce the concentration of an enzyme-bound factor (possibly NADH) and that the lag prior to decay represented the time required for the enzyme-bound component to dissociate.

NADH dehydrogenase activity was stimulated by NaCl, although considerable activity was present in the absence of added salt. This type of salt response has been observed with other enzymes obtained from halophilic bacteria and may suggest that extraordinarily high concentrations of salt may not be necessary to activate all of the enzymes obtained from extremely halophilic bacteria. However, this response was much different from that reported for the NADH dehydrogenase obtained from *Halobacterium salinarium* and the menadione reductase obtained from *H. cutirubrum*. The enzyme from *H. salinarium* was completely inactive at salt concentrations less than 1.0 M NaCl. At salt concentrations greater than 1 M, the response to increasing salt concentrations appeared sigmoidal. This kind of sigmoidal response has been reported to be due to preincubating halophilic enzymes at suboptimal concentrations of salt, thus measuring the residual activity following inactivation of the enzyme. Since no experimental details were described, it cannot be determined whether this explanation could account for the differences in the salt response exhibited by the enzymes from AR-I and *H. salinarium*.

The menadione reductase from *H. cutirubrum* also responded differently than the NADH dehydrogenase from AR-I. Both enzymes were maximally active at about the same salt concentration. However, the *H. cutirubrum* menadione reductase was inactive in the absence of added salt. Since the activity reported for *H. cutirubrum*
menadione reductase was obtained from initial rate determinations, this disparity represented a difference in the activity of the enzymes to salt. In addition, the menadione reductase from *H. cutirubrum* was maximally active in 1.5 M NaCl while the NADH dehydrogenase from AR-I, using menadione as an acceptor, was maximally active in 0.5 M NaCl and considerable activity was observed in the absence of added salt (Hochstein, L. I. and Dalton, B. P., unpublished). These differences, as well as differences in molecular weight, pH optima, pH stability, and light sensitivity (Lanyi, J. K., personal communication) suggest that these NADH dehydrogenases (even though membrane-bound and analogous in function) may be constructed along sufficiently different lines so that the molecular forces responsible for structural integrity, as well as enzyme activity, may be radically different. A survey of the NADH dehydrogenases from a wide range of halophilic bacteria, and a comparison of their kinetic properties may shed light on the significance of this apparent discrepancy.

REFERENCES
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