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Studies on a Gram-Positive Hydrogen Bacterium, *Nocardia opaca 1 b*

III. Purification, Stability and Some Properties of the Soluble Hydrogen Dehydrogenase

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Abstract. Nocardia opaca strain *1 b* has a NAD-dependent hydrogenase (hydrogen dehydrogenase). The enzyme has been purified from autotrophically grown cells and tested for optimal assay conditions and stability. The purification procedure involved protamine sulfate treatment, ammonium sulfate precipitation, and separation by DEAE-cellulose and Sephadex G-200 chromatography and resulted in a 63-fold increase of specific activity at a 11.7% enzyme recovery. The final specific activity was 103 μmoles H_2/min · mg protein.

The purified enzyme was dependent on nickel and magnesium ions at 0.5 and 5.0 mM concentrations, respectively, as well as flavin mononucleotide at a 5—10 μM concentration. Straight enzyme kinetics were achieved by preincubating the enzyme in the presence of $NADH_2$.

A high stability of the enzyme was observed in 0.1 M potassium phosphate buffer, pH 6.5, in the presence of 0.5 mM nickel and 5 mM magnesium ions under hydrogen atmosphere. Even under air the enzyme was remarkably stable, although less than under hydrogen.

From double reciprocal plots of substrate saturation curves the Michaelis-Menten constants were calculated: For saturating NAD-concentration the K_m was 0.063 mM H_2 and for saturating hydrogen concentration the K_m was 0.123 mM NAD.

Key words: NAD-Dependent Hydrogenase — Hydrogen Dehydrogenase — *Nocardia opaca* Strain *1 b* — Hydrogen Bacteria — Chemolithoautotrophic Bacteria — Gram-Positive Hydrogen Bacteria.

The reduction of NAD by molecular hydrogen has been observed with extracts of various bacteria such as *Hydrogenomonas eutropha* (Bovell, 1957; Wittenberger and Repaske, 1961; *H 16:* Eberhardt, 1966), *H. ruhlandii* (Packer and Vishniac, 1955), *Pseudomonas saccharophila*, *Clostridium kluyveri* (Korkes, 1955), *C. pasteurianum* (Shug *et al.*, 1954; Peck and Gest, 1954), and *Chlorobium* (Arnon, 1961). While the NAD-reducing systems in *C. kluyveri* and *H. eutropha* require flavin, the enzyme from *H. ruhlandii* did not respond to the addition of flavins (Bernstein and Vishniac, 1959).

The crude extract of autotrophically grown cells of *Nocardia opaca 1 b* catalyzed the reduction of NAD and oxygen as well as of artificial hydrogen-acceptors like methylene blue, benzylviologen and methylviologen by molecular hydrogen (Aggag and Schlegel, 1973). The enzyme hydrogenase was absent from fructose grown cells and its specific activity reached high values during autotrophic growth. Different fractions obtained by fractional centrifugation of the crude extract reduced NAD. This result was in contrast to the experiences made with other hydrogen bacteria. E.g. while the soluble fraction of *H. eutropha H 16* contained a NAD-reducing hydrogenase (hydrogen dehydrogenase) the particles reduced methylene blue or oxygen, but not NAD (Eberhardt, 1966). The hydrogen dehydrogenase located in the soluble fraction of cell-free extracts of *N. opaca 1 b* was studied more closely. A partially purified preparation was used for stability studies and a more highly purified preparation served for kinetic experiments.

Materials and Methods

Nocardia opaca strain *1 b* was grown autotrophically in 10-l quantities as described by Aggag and Schlegel (1973). The cells were harvested, washed twice with and suspended in 0.1 M K-phosphate buffer, pH 6.5. The extracts were prepared by sonic vibration for 30 sec/ml cell suspension. A sonifier (20 KH, 600 W, Schoeller and Co., Frankfurt, Germany) was used in combination with a cooled (Ultrakryomat TK 30 D, Meßgerät Lauda) flow-through vessel of 30 ml capacity. The temperature was maintained between $+2°$ C and $+10°$ C. The disrupted cell suspension was centrifuged at $10000 \times g$ for 30 min to remove unbroken cells and cell debris. The cell-free extract was then centrifuged at $140000 \times g$ for 3 hr.

Enzyme Purification. All purification steps were carried out at 4° C. The buffers used for the elution of the extract during chromatography on Sephadex G-25, G-200 and DEAE-cellulose were maintained saturated with hydrogen. This was achieved by flushing the buffer continuously with oxygen-free hydrogen. All the buffers used in the extraction process were supplemented with $MgSO_4$ (5 mM) and $NiCl_2$ (0.5 mM).

The purification of the soluble hydrogenase involved the steps in Table 1.

Protamine Sulfate Treatment. Protamine sulfate (1% w/v) was added dropwise to the stirred extract containing approximately 25 mg protein/ml in a proportion of 1 mg/10 mg of the extracted protein. The precipitate was removed by centrifugation at $20000 \times g$ for 30 min and discarded.

Ammonium Sulfate Fractionation. The supernatant from the last step was fractionated with ammonium sulfate. The pH of the extract was adjusted to 6.5 with the addition of ammonia solution. The fraction between 35—55% saturation was collected and dissolved in 10 mM K-phosphate buffer, pH 6.5. The desalting of the ammonium sulfate fraction was accomplished by passing it through a column of Sephadex G-25 gel, previously equilibrated with 10 mM phosphate buffer, pH 6.5.

Chromatography on DEAE-Cellulose. The desalted protein fraction was then applied to a 2.5×15 cm column of DEAE-cellulose (Whatman DE-52 microgranular grade) which had been equilibrated with 10 mM phosphate buffer, pH 6.5. The extract adsorbed on the top of the resin column was washed with 100 ml of 0.1 M phosphate buffer. The enzyme was eluted with a linear gradient between 0.1

Table 1. Purification and properties of soluble hydrogenase

Step	Total protein (mg)	Specific activity (mE/mg protein)	Purification	Recovery of activity %
Crude extract	1890	1630	1.0	100.0
Protamine sulfate supernatant	1638	1956	1.2	104.0
Ammonium sulfate 35—55% (desalted with Sephadex G-25)	356	5705	3.5	66.0
DEAE-cellulose	27.6	39094	24.0	35.0
Sephadex G-200	3.49	103179	63.3	11.7

and 0.4 M K-phosphate buffer each 500 ml, pH 6.5. The flow rate was 70 ml/hr, and 6.8 ml fractions were collected. The most active fractions were combined and concentrated to about 3 ml by ultrafiltration in an Amicon diaflo cell.

Chromatography on Sephadex G-200. The concentrated effluent from the DEAE-cellulose column was layered on top of a Sephadex G-200 column (fine grade 2.6× 72 cm) and eluted with 0.1 M phosphate buffer. The flow rate was approximately 12 ml/hr, and fractions of 1 ml were collected. The most active fractions were combined and stored under H_2-atmosphere in small aliquots at −20° C until use. The final preparation had a 63-fold increased specific activity compared with the crude extract.

Determination of Hydrogenase Activity. Hydrogenase activity was measured spectrophotometrically by following the rate of NAD-reduction by molecular hydrogen at 365 nm (Zeiss PL 4) at 25° C in 1-cm cuvettes. The cuvettes were stoppered with rubber caps permitting the use of hypodermic needles to flush the cuvettes with hydrogen. All the solutions used in the assay were maintained saturated with hydrogen by flushing them continuously with a stream of O_2-free hydrogen. The assay mixture contained 125 μmoles of triethanolamine buffer (pH 8.0), 5 mM $MgSO_4$, 0.5 mM $NiCl_2$ and 2.0 μmoles of NAD. The reaction was started with the addition of the enzyme preparation. The initial rate was proportional to the enzyme concentration up to an absorbancy change of 0.3/min. The final reaction mixture had a total volume of 3.0 ml. Controls containing argon instead of hydrogen were always inactive. A unit of enzyme activity was defined as 1.0 μmole NADH formed per min, and specific activity as μmole NADH/min · g protein. Protein was estimated by the Lowry method (Lowry *et al.*, 1951).

Chemicals were those listed by Aggag and Schlegel (1973).

Results

The hydrogenase activity measured as the rate of NAD-reduction decreased considerably during the purification process. Therefore, the tests for stability and cofactors were made using the effluent of the DEAE-cellulose column (15—24-fold purified enzyme).

Table 2. Effect of buffer on the rate of NAD-reduction by H_2

Buffer, pH 8.0	ΔE_{365} per min at buffer concentration (M)				
	0.5	0.1	0.05	0.01	0.001
Triethanolamine	0.040	0.160	0.205	0.190	0.130
Tris-HCl	0.095	0.180	0.155	0.145	0.105
Potassium phosphate	0.230	0.215	0.195	0.145	0.120

Enzyme was diluted 1:10 in 0.05 M phosphate buffer. For each assay 0.1 ml of this dilution was preincubated with $MgSO_4$, $NiCl_2$ and FMN as described in "Materials and Methods". After the preincubation period 2.5 ml of the tested buffer were added. Reaction was started by addition of 2 μmoles NAD.

Optimum Assay Conditions and Stability

Effect of pH and Buffer on Activity. The pH-optimum for NAD-reduction by a 15-fold purified hydrogenase preparation was between 8.0 and 8.2 (Fig. 1) for both TEA and Tris-HCl buffers. Within this pH-range the rate of NAD-reduction was influenced by the buffer itself as well as by its concentration. As indicated in Table 2, the hydrogenase activities with phosphate, TEA, and Tris-HCl buffers were relatively low at 0.001 M. With TEA and Tris-HCl, the rate of NAD-reduction increased with the concentration of the buffer up to a certain concentration. The highest activities were obtained at 0.05 M TEA and 0.1 M Tris buffer. Higher concentrations of Tris and TEA buffers caused significant irreversible inactivation of the enzyme. On the other hand the rate of NAD-reduction increased with the phosphate concentration.

Fig. 1. Effect of pH on the activity of a 15-fold purified preparation of hydrogen dehydrogenase from *Nocardia opaca 1 b*. The enzyme was diluted 1:10 in the indicated buffer. For each assay 0.1 ml (72.0 μg protein) of the enzyme dilution was preincubated with Mg^{++}, Ni^{++} and FMN as described in "Materials and Methods". After the preincubation time 125 μmoles of the tested buffer were added. The test was started with the addition of 2 μmoles NAD. Symbols: ▵ TEA buffer, ○ Tris-HCl buffer

Fig. 2. Effect of metals and FMN on the rate of NAD-reduction by H_2. The enzyme was diluted 1:20 in 0.05 M phosphate buffer, pH 7.0. For each assay 0.1 ml (36.0 μg protein) was used. The assay mixture contained 5 mM Mg^{++}, 0.5 mM Ni^{++} and 0.01 mM FMN where indicated. The assay was done in TEA buffer as described under "Materials and Methods"

Fig. 3. Effect of pH on stability of NAD-reducing system. The enzyme was diluted 1:20 in 0.05 M phosphate buffers of the indicated pH values and the enzyme dilutions were kept for 3 days at 4° C under H_2-atmosphere. For the assay 0.1 ml (36 μg protein) was removed and NAD-reduction was determined as in "Materials and Methods"

Phosphate buffer, however, has the disadvantage of forming slight precipitates with the metals added to the assay mixture at pH 8.0.

Effect of Metals on Activity. The rate of NAD-reduction by hydrogen in the crude extract was low and did not follow linear kinetics when the

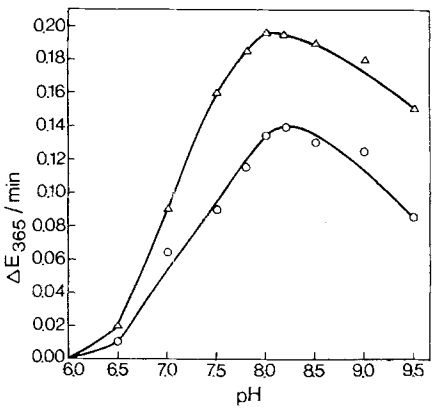

Fig. 1

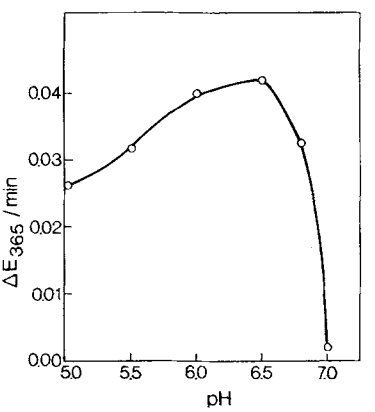

Fig. 3

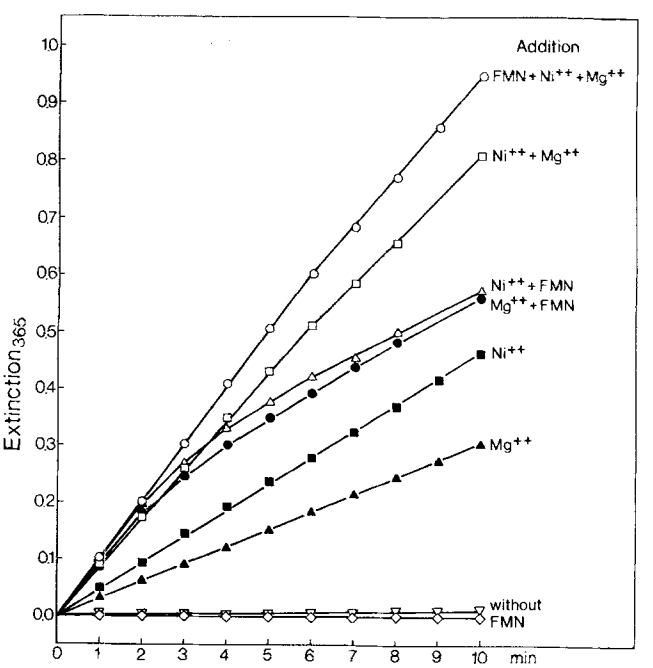

Fig. 2

Table 3. Effect of flavins on the rate of NAD reduction by H_2

Additions	Concentration (mM)	ΔE_{365}/5 min
none		0.005
FMN	0.01	0.005
FAD	0.01	0.006
Riboflavin	0.01	0.007
$NiCl_2$	0.5	0.200
$NiCl_2$ + FMN	0.5 + 0.01	0.350
$NiCl_2$ + FAD	0.5 + 0.01	0.185
$NiCl_2$ + riboflavin	0.5 + 0.01	0.290

The enzyme was diluted 1:20 in 0.05 M phosphate buffer, pH 7.0. For each assay 0.1 ml of the diluted enzyme (36.0 μg protein) was preincubated for 3 min with the above substances. The assay was done in TEA buffer as described in "Materials and Methods".

activity was assayed in the absence of metals. Hydrogen-dehydrogenase activity, if any, was detected only when a relatively large amount of the crude extract was used (about 1 mg protein/cuvette). Moreover, when the extract was passed through Sephadex G-25 gel, about 50% of the hydrogenase activity were lost. These observations drew the attention to look for certain metal(s) which may be necessary for the reactivation of the enzyme. All the metals included in the growth medium as trace elements were tested. Only Ni^{++}, Mg^{++}, Co^{++}, and Mn^{++} affected a reactivation, Fe^{++}, Zn^{++} and molybdate were inactive.

The NAD-reduction, however, followed linear kinetics over 5—10 min with Ni^{++} and Mg^{++} only when those were added in concentrations of 0.5 mM and 5 mM, respectively, to the assay mixture. Although Mn^{++} and Co^{++} stimulated the NAD-reduction at the beginning of the reaction, the enzyme activity decreased rapidly after about 1—3 min. From Fig. 2 it can be seen that when the diluted enzyme preparation (1:20 in phosphate buffer) was assayed in absence of metals, the NAD-reduction was scarcely demonstrable. On the contrary, when the same enzyme dilution was assayed in the presence of Ni^{++} and Mg^{++} more than 50-fold activation was achieved.

Effect of Flavins on Activity. Table 3 shows the response of the hydrogenase to 3 flavins, flavin mononucleotide (FMN), flavin adenosine dinucleotide (FAD) and riboflavin when added alone or together with nickel to the assay mixture. None of the tested flavins in the concentrations indicated could stimulate the NAD-reducing system. Only when FMN or riboflavin were added in combination with magnesium or nickel ions, the NAD-reducing system was activated. The response of hydro-

Table 4. Effect of sodium, potassium and ammonium salts on the rate of NAD reduction by H_2

Additions	Concentration (M)	ΔE_{365}/5 min	Additions	Concentration (M)	ΔE_{365}/5 min
none		0.29	KCl	0.02	0.315
				0.05	0.325
$(NH_4)_2SO_4$	0.02	0.300		0.10	0.310
	0.05	0.350			
	0.10	0.425	Na_2SO_4	0.02	0.260
				0.05	0.190
NH_4Cl	0.02	0.290		0.10	0.170
	0.05	0.365			
	0.10	0.360	NaCl	0.02	0.225
				0.05	0.180
K_2SO_4	0.02	0.315		0.10	0.165
	0.05	0.385			
	0.10	0.445			

The enzyme was diluted 1:30 in 0.05 M TEA buffer, pH 7.0. For each assay 0.1 ml (24.0 μg protein) was used. The enzyme was preincubated with the above salts in addition to magnesium and nickel ions and FMN as described in "Materials and Methods".

genase to riboflavin was less than that to FMN. FAD did not exert any activating effect. The highest rate of NAD-reduction was achieved by FMN at a concentration between 5 and 10 μM. The rate of NAD-reduction was shown to be slightly influenced by the sequence of addition of FMN and the divalent metals to the enzyme preparation, too.

Effect of Glutathione on Activity. Addition of 10 mM glutathione to the enzyme diluting medium increased the rate of NAD-reduction to about 20% compared to the enzyme activated only by hydrogen. When glutathione was added to the assay mixture, the rate of NAD reduction was slightly increased up to a concentration of 0.1 mM. Higher concentrations than 0.1 mM glutathione in the assay mixture inactivated the enzyme significantly. Other reducing agents, such as mercaptoethanol and dithiothreitol could not be used due to the formation of colored compounds with the metals present in the assay mixture.

Effect of Sodium, Potassium, and Ammonium Salts on Activity. Table 4 shows the effect of ammonium, sodium, and potassium cations as well as the chloride and sulfate anions on the hydrogenase activity. While potassium, ammonium and sulfate ions stimulated the rate of NAD-reduction, sodium ions inhibited the enzyme and the inhibition

Table 5. Effect of the kind of buffer on the stability of hydrogenase

Buffer 0.05 M, pH 6.5	Loss of hydrogenase activity (%)	
	after storage for 3 days at 4° C	after heating at 45° C for 10 min
TEA	90	95
Tris-HCl	87	90
Phosphate	40—45	30

The enzyme was diluted 1:20 in the indicated buffers. Enzyme dilution was stored for 3 days at 4° C under hydrogen atmosphere. For each assay 0.1 ml (36.0 μg protein) was removed for hydrogenase assay as described under "Materials and Methods".

appeared to be related to the sodium concentration. Chloride ions caused no activation.

Factors Affecting Stability of Hydrogenase; pH and Buffer. The effect of pH on enzyme activity was determined in diluted preparations in phosphate buffer after 3 days storage at 4° C under a hydrogen atmosphere. Fig. 3 shows that the optimal pH for stability of the NAD-reducing system was 6.5. At neutral and alkaline pH-values the enzyme lost almost completely the NAD-reducing property after 3 days. It was also found that the stability was influenced by the buffer used. When the enzyme was diluted in 0.05 M Tris or TEA buffers at pH 6.5 it lost more than 90% of its activity within 3 days. On the other hand 55—60% of hydrogenase activity was retained in the preparations diluted in phosphate buffer.

On heating for 10 min at 45° C the enzyme dilutions prepared in Tris or TEA lost more than 90% of their NAD-reducing activities while those diluted in K-phosphate buffer retained more than 70% of their activities (Table 5).

The effect of magnesium and nickel ions, FMN and glutathione on the stability of the enzyme to storage under hydrogen and air atmospheres was investigated in diluted enzyme preparations. The enzyme was diluted 1:20 in 0.1 M K-phosphate buffer, pH 6.5 in the presence of the substances indicated in Table 2. The data show that magnesium together with nickel in concentrations of 5 mM and 0.5 mM, respectively, exert a pronounced stabilizing effect on the NAD reducing system. In the presence of magnesium and nickel about 90% and 60% of the enzyme activities were retained after 3 days storage under hydrogen and air atmospheres, respectively. Glutathione in 10 mM exerted no additional advantage on that effect produced by the divalent salts alone. Unexpectedly it was found that when FMN was added in 5 μM concentration to the enzyme dilution together with magnesium and nickel ions,

Table 6. Hydrogenase activity with methyl- and benzylviologen as H-acceptor

H-acceptor	Specific activity (E/mg protein)
NAD	101
Benzylviologen	88
Methylviologen	26

Assay mixtures contained in 3 ml: 5 mM $MgSO_4$, 0.5 mM $NiCl_2$, 125 μmoles TEA (pH 8.0), H_2-acceptors (methylviologen 10 mM, benzylviologen 10 mM or NAD 0.67 mM). The mixtures were bubbled with hydrogen gas for 5 min at 25 °C. Reaction was started by the injection of the enzyme into the H_2-saturated mixture.

the NAD-reducing activity was almost completely lost when the enzyme dilution was kept under H_2 atmosphere for 3 days. On the other hand when a similar enzyme preparation was kept for 3 days under air atmosphere, about 50% of the enzyme activity was still measured. It seems that the hydrogenase enzyme in the reduced form is more susceptible to the irreversible inactivation caused by FMN than when it is in the oxidized form.

Acceptors, Inhibitors and Kinetics

Acceptors. For studying the acceptor specificity and kinetics of the hydrogenase from *Nocardia opaca 1 b* the 63-fold purified enzyme preparation was used. This preparation was still able to catalyze the reduction of NAD, benzyl- and methylviologen with hydrogen (Table 6). The ratio of activities for benzylviologen and methylviologen to NAD were 0.87 and 0.26, respectively. On the other hand the reduction of NADP, FMN and methylene blue under the same experimental conditions as described under Table 6 was not observed. In contrast to the powerful reduction of methylene blue by the crude extract and the soluble fractions (Aggag and Schlegel, 1973), the purified enzyme could not reduce methylene blue unless catalytic amounts of NAD or NADH were added to the assay mixture (Table 7). In case of added NADH, the reduction of methylene blue was almost the same under hydrogen or nitrogen atmospheres. The optimum pH for methylene blue reduction was 7.0, and the activity was not influenced by the addition of magnesium and nickel to the assay mixture. All the above findings may indicate that the reduction of methylene blue was catalyzed by a diaphorase rather than hydrogenase enzyme. Similarly, the reduction of FMN by molecular hydrogen was not effected with the purified enzyme. In the presence of NAD, however, the reduction of FMN occurred slowly, visible by the disappearance of the yellow colour.

Table 7. Methylene blue reduction

Additions	Gas atmosphere	ΔE_{365} per min
—	H_2	0
$NADH_2$	H_2	0.35
$NADH_2$	N_2	0.33
NAD	H_2	0.30
NAD	N_2	0

Assay mixtures contained in 3 ml: 5 mM $MgSO_4$, 0.5 mM $NiCl_2$, 125 μmoles TEA (pH 7.0), 134 nmoles methylene blue, 0.2 μmole of NADH or NAD (where indicated). The mixtures were bubbled with hydrogen gas for 5 min at 25° C. Reaction was started by the injection of the enzyme into the H_2-saturated mixture.

Table 8. Effect of inhibitors on the rate of NAD-reduction by H_2

Inhibitor	Inhibitor concentration (mM)	Inhibition (%)	
		Inhibitor mixed in air	Inhibitor mixed with NADH under H_2
Iodoacetate	100	36	56
p-Chloromercuribenzoate	0.0001	30	
	0.0002	52	
	0.002	72	90
	0.02	100	
p-CMB + glutathione	0.02 + 1.0	23	
KCN	1.0	33	19
Na_2S	0.1	10	

For incubating the enzyme with the inhibitor in air, the enzyme (4.2 μg protein) was preincubated in air atmosphere for 5 min at 25° C with the inhibitor; 25 μmoles TEA buffer, pH 8.0, in a total volume of 0.5 ml. For incubating the inhibitor with the enzyme in the reduced form, the enzyme (4.2 μg protein) was preincubated with 0.2 μmoles NADH, 25 μmoles TEA buffer, pH 8.0 for 5 min at 25° C under hydrogen. The reaction was started by the addition of 100 μmoles H_2-saturated TEA buffer, pH 8.0 and 2.0 μmoles NAD to the preincubation mixture, total volume 3.0 ml.

Using large amounts of less purified enzyme preparation, FMN-reduction could be demonstrated. The difference spectra of air minus hydrogen or argon minus hydrogen showed a pronounced absorption at 450—455 nm.

Effect of Inhibitors on Hydrogenase. Table 8 shows the effect of different inhibitors on NAD-reduction by molecular hydrogen. Inhibition by iodoacetate was low and at a concentration of 0.1 M only 36% inhibition of hydrogenase was demonstrated. *p*-Chloromercuribenzoate was strongly inhibitory and suppressed the hydrogenase activity completely at

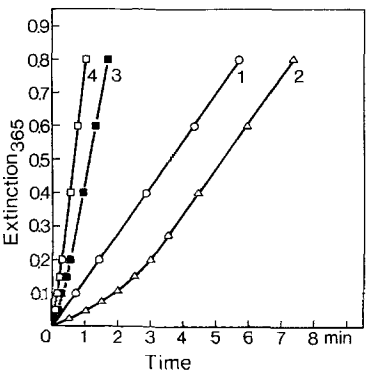

Fig. 4. Effect of enzyme concentration and NADH addition on the time course of NAD-reduction by 63-fold purified hydrogenase. Curve *1:* The enzyme (1.4 μg protein) was preincubated in the presence of 0.5 mM $NiCl_2$, 5.0 mM $MgSO_4$ and 0.2 μmole NADH under hydrogen for 5 min. After the incubation 125 μmoles H_2-saturated TEA buffer, pH 8.0 were added, and the reaction was started by adding 2 μmoles NAD; final volume 3.0 ml. Curve *2:* as in 1 except no NADH. Curves *3* and *4:* as in 2, but higher enzyme concentration, 7.0 and 9.8 μg protein

0.02 mM. The inhibition by *p*-CMB was strongly reversed through the addition of excess glutathione.

The inhibitory effects of iodoacetate and *p*-CMB were higher when they were added to the enzyme in the reduced form than when the enzyme was preincubated with the inhibitors in air atmosphere. The obtained results indicate the presence of sulfhydryl groups in hydrogenase which are essential for enzymic activity.

Sulfide in a concentration of 0.1 mM caused only slight inhibition of hydrogenase. In contrast to sulfhydryl inhibitors, cyanide exerted a stronger inhibitory effect on the oxidized enzyme compared to the reduced enzyme.

Kinetics of NAD-Reduction. In contrast to crude cell-free extracts or the 15-fold purified preparation the 63-fold purified preparation reduced NAD with a lag-phase (Fig. 4, curve 2). When the enzyme was preincubated in the presence of NADH, no lag-phase was observed (curve 1). Preincubation of the enzyme for 5 min with hydrogen only under the assay conditions without NAD did not substitute for the NADH preincubation. The lag-phase could also be decreased by increasing the enzyme concentration (curves 3 and 4).

On the other hand, considerable reactivation of the hydrogenase was achieved, when the original enzyme solution was evacuated and flushed with oxygen-free hydrogen and then incubated under H_2 for at least 1 hr at 4° C.

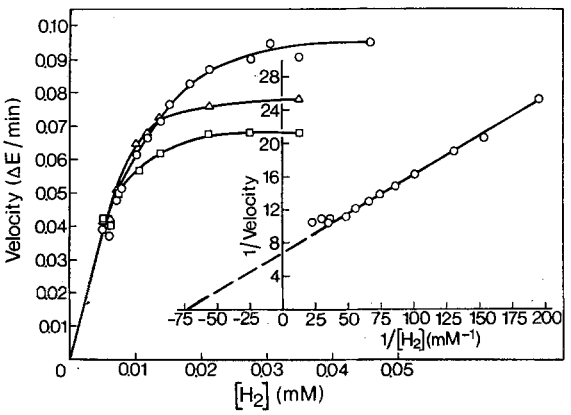

Fig. 5. Substrate(H_2)-saturation curves of the 63-fold purified hydrogenase preparation. The assay mixture contained in a final volume of 3.00 ml: 0.5 mM $NiCl_2$, 5.0 mM $MgCO_4$, 125 μmoles TEA buffer, pH 8.0, and 0.167 (□), 0.33 (△), and 0.67 (○) mM NAD. The buffer was saturated with O_2-free gas mixtures of argon and hydrogen at varying ratios. The reaction was started by the addition of 1.4 μg enzyme protein to the assay mixture. Lineweaver-Burk plot inserted

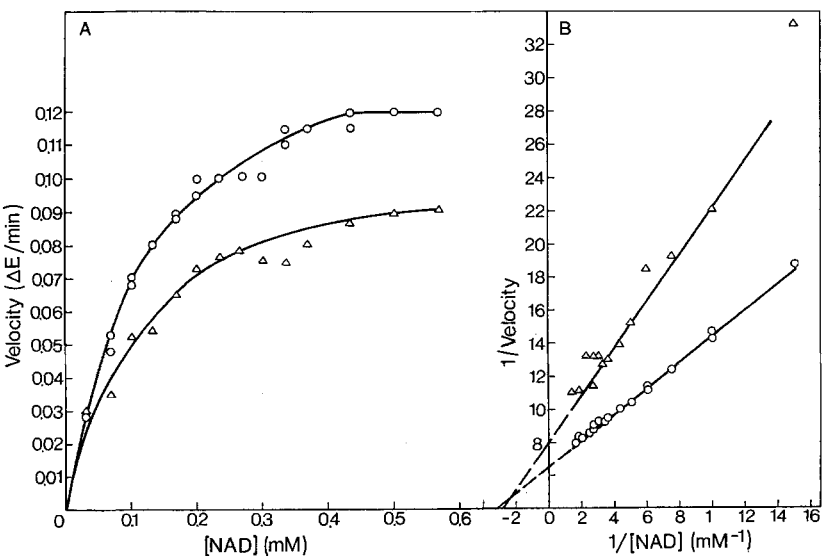

Fig. 6 A and B. Substrate(NAD)-saturation curves of the 63-fold purified hydrogenase preparation. The assay mixture was equal to that in Fig. 5, except two different hydrogen concentrations were used: 0.042 (△) and 0.6 (○) mM H_2. The NAD-concentration was varied. (A) Substrate saturation curves. (B) Lineweaver-Burk plot

Substrate-saturation curves for H_2 were measured by varying the concentration of H_2-saturated TEA buffer in N_2-saturated buffer and at three different NAD-concentrations (Fig. 5). Hyperbolic curves were obtained. From the double reciprocal plot the K_m-value for saturating concentration of NAD was determined to be 0.063 mM hydrogen.

The NAD-saturation curve (Fig. 6) was measured in the presence of two hydrogen concentrations (0.042 and 0.16 mM). From the double-reciprocal plots a K_m-value of 0.123 mM NAD for saturating hydrogen concentration was obtained.

Discussion

The influence of metal ions on the activity and stability of hydrogenase preparations from different organisms have been frequently reported. Nickel has not been previously shown to have such an essential role in the stimulation of hydrogenase activity. A specific nickel requirement for the chemoautotrophic growth of *Hydrogenomonas H 16* and *H 1* was observed by Bartha and Ordal (1965). However, a requirement of nickel for the synthesis or activation of hydrogenase or for the Knallgas reaction could not be demonstrated. The authors' suggestion tends to the participation of nickel in some reactions involved in CO_2 fixation by growing cells.

The rate of NAD-reduction by the purified hydrogen dehydrogenase from *Nocardia opaca 1 b* was manyfold increased by the addition of nickel and magnesium. The metal requirement for NAD-reduction is apparently not specific for nickel, since magnesium, cobalt, and manganese, though in higher concentrations, can partially substitute for nickel.

Iron or other heavy metals are indispensible for dehydrogenase. The prosthetic group of hydrogenase is assumed to be an organometallic complex and the function of the metal is concerned with the initial binding with hydrogen.

The kinetics of hydrogenase and the role of molybdenum on the activity of the enzyme in *Clostridium pasteurianum* were subject to investigations by Tamiya and Miller (1963) and Kleiner and Burris (1970). The former proposed an asymmetrical hydrogen site for accepting a proton and a hydride ion arising from the heterolytic dissociation of hydrogen. They suggested that the site concerned with the acception of the hydride ion is a metal, since many metal ions in solution are capable of activating hydrogen by forming hydride.

When the hydrogenase preparation was kept under air atmosphere in neutral or alkaline media, a rapid and irreversible inactivation of the enzyme was noticed. Irreversible denaturation of the enzyme was also caused by high concentrations of Tris or triethanolamine buffer. A similar

effect of Tris buffer was observed with the enzyme from *Pseudomonas ruhlandii* (Bone *et al.*, 1963). The irreversible denaturation of the hydrogenase system of *Nocardia* probably results from oxidations of reactive sulfhydryl groups in the enzyme. Participation of sulfhydryl groups is indicated by the effect of *p*-Chloromercuribenzoate.

That maximal rate of NAD-reduction by the hydrogenase is reached after a lag-phase has been observed severalfold. The removal of the lag-phase by preincubating the enzyme with $NADH_2$ or H_2 can be explained as in the case of hydrogen dehydrogenase from *P. ruhlandii* (Bone, 1963), *i.e.* by the existence of two enzymic forms, one being inactive which is in equilibrium with the active form. On reduction of the active form, the inactive form is slowly converted into the active form. The partial inactivation of the enzyme may be ascribed to the oxygenation of the enzyme (Fisher *et al.*, 1954) or to the removal of certain cofactors necessary for the activation of hydrogenase.

The hydrogen dehydrogenase purified from the soluble fraction of the cell-free extract had a high degree of specificity. It did not reduce methylene blue in the absence of NAD. Similarly, the 45-fold purified soluble hydrogenase from *Hydrogenomonas eutropha H 16* could not reduce methylene blue in the absence of catalytic amounts of NAD, although the dye was efficiently reduced by the crude extract (Pfitzner *et al.*, 1970). The results obtained with the *H 16* and the *1 b* enzymes indicate that the reduction of methylene blue was catalyzed by a diaphorase in addition to hydrogenase enzyme. However, it was astonishing to observe the reduction of methyl- and benzylviologen, although at a lower rate compared to NAD as H-acceptor.

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