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NADH- and H_2-Oxidation in Hydrogen Bacteria Studied by Respiratory Chain Inhibitors

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Summary. The oxidation of hydrogen and NADH by membrane fractions of two autotrophically grown hydrogen bacteria, *Pseudomonas facilis* and strain *14 g*, both lacking a hydrogen dehydrogenase, was studied by difference-spectrophotometric and manometric methods. The spectrophotometric data did not support the existence of two separate electron transport pathways for both the substrates. However, from the effect of rotenone, antimycin A, BAL, and HQNO on the oxygen uptake rate with H_2 or NADH as substrates, separate pathways could be proposed: in strain *14 g* at least to *cytochrome b* and in *P. facilis* at least to *cytochrome c*.

The involvement of cytochromes in hydrogen oxidation and the effect of respiratory chain inhibitors has been described in *Pseudomonas ruhlandii* (Packer, 1958). ATP-formation coupled to H_2 oxidation has been reported for *Hydrogenomonas* strain *H 20* (Bongers, 1967). As indicated in several papers published more recently, the hydrogen oxidizing system of hydrogen bacteria is similar to the respiratory chain of heterotrophic bacteria.

All hydrogen bacteria studied so far are able to grow heterotrophically as well. They, therefore, ought to contain an ordinary NADH-oxidase system. At least some strains of the hydrogen bacteria do not contain a NAD-reducing hydrogen dehydrogenase (Eberhardt, 1965; Schneider *et al.*, 1973). A reverse electron transport is, therefore, likely to occur under autotrophic conditions. The relations between the NADH and the H_2 oxidizing systems were repeatedly investigated. From the ineffectiveness of a NADH-trap on hydrogen oxidation by cell-free preparations of *Hydrogenomonas H 20* it was concluded that NAD is not involved in the oxy-hydrogen reaction and that H_2 and NADH are oxidized via different pathways (Bongers, 1967). An opposite conclusion was drawn from the inhibition of the oxy-hydrogen reaction

Abbreviations. BAL = 2,3-Dimercaptopropanol; HQNO = 2-n-Heptyl-4-hydroxyquinoline-N-oxide; TTFA = Thenoyltrifluoroacetone.

by a NADH-trap in *Hydrogenomonas eutropha* (Ishaque and Aleem, 1970). Two separate electron transport pathways from NADH and from H_2 to *cytochrome c* have been proposed on the basis of inhibition experiments employing purified membranes of *Hydrogenomonas eutropha H 16* (Pfitzner, 1969). This study deals with the same question and with *Pseudomonas facilis* and a coryneform bacterium strain *14 g*, both bacteria lacking a NAD-dependent hydrogen dehydrogenase (Schneider *et al.*, 1973).

Materials and Methods

Organisms. Pseudomonas facilis (ATCC 17695) and the coryneform bacterium strain *14 g* (Schneider *et al.*, 1973) were used. Growth conditions, the preparation of the membrane fraction, protein determination, and measurement of reduced minus oxidized difference spectra have been described by Bernard *et al.* (1973). For the measurement of difference spectra after reduction by hydrogen, a concentrated membrane suspension was mixed with the 10 or 20-fold volume of buffer saturated by hydrogen within the cuvette. If NADH was used as the reducing agent, it was added in the dry state.

Manometric Techniques. The activities of the hydrogen- and NADH oxidases were measured manometrically using a round Warburg apparatus (Braun, Melsungen). The conditions were: Temperature 25° C, 110 strokes per min, amplitude 8 cm, reaction volume 2.5 ml; 0.1 M potassium phosphate buffer, pH 6.8; gas atmosphere 95% N_2 + 5% O_2 or 95% H_2 + 5% O_2, resp. Gas uptake rates were calculated from the 5th to 45th min after tipping in the substrate, or starting shaking. Inhibitors (exempt KCN) were dissolved in ethanol and pipetted into the main compartment (containing the membrane suspension) immediately prior shaking. Controls made sure that the ethanol was in equilibrium with the gaseous phase and did not contribute to pressure changes.

Chemicals. Commercial chemicals of high purity and pro analysis of E. Merck, Darmstadt, were used. Rotenone was obtained from The British Drug House, Ltd.; Antimycin A and BAL from Serva Entwicklungslabor, Heidelberg; HQNO from Sigma Chemical Company, St. Louis; NADH from Boehringer, Mannheim; TTFA from E. Merck, Darmstadt.

Results

The amount of cytochromes and flavoproteins present in the membrane fraction of autotrophically grown cells was measured by difference spectrophotometry (dithionite reduced minus oxidized spectra). If NADH or H_2 were employed as reducing agents, the extent of reduction was less (Table 1). The incomplete reduction may be due to a partial damage of the electron transport system by sonication. The quantitative analysis of the data was restricted to *cytochromes b* and *c* since the determination of *cytochrome a* was less accurate.

The reduction of *cytochrome b* by NADH was rather low. In the membrane fraction of *Pseudomonas facilis* no reduction of *cytochrome b* by NADH was detected; in strain *14 g* it was reduced to a lesser degree than *cytochrome c*. If NADH and H_2 were administered concomitantly, the extent of reduction was approximately equal to that after addition

Table 1. Reduction of membrane-bound redox carriers by physiological reducing agents, measured by room temperature difference spectrophotometry. Data indicate μmoles/g protein

Organism	Reducing agent	*Cyt. a*	*b*	*c*	Flav.
Pseudomonas facilis	Dithionite	+	0.62	0.60	1.26
(3 mg membrane	H_2	+	0.37	0.39	0.52
protein/ml)	NADH	+	[a]	0.39	0.43
	NADH + H_2	+	0.39	0.44	0.52
Strain *14 g*	Dithionite	+	0.52	0.36	0.86
(1.2 mg membrane	NADH	+	0.17	0.27	0.16
protein/ml)	NADH + H_2	+	0.24	0.27	0.16

[a] In room temperature spectra not detectable.

of only H_2. If NADH and H_2 would be oxidized by strictly separate pathways, one would have expected an additive effect.—Strain *14 g* contained more *cytochrome b* than *cytochrome c*. The percentage degree of reduction was less in the former than in the latter, even in the presence of both reducing agents, NADH and H_2.

The question whether two separate electron transport pathways are used for the oxidation of NADH, and H_2 was checked by inhibition experiments. The rate of oxygen uptake was measured manometrically and immediately after preparing the membrane suspension. The inhibitors were added as ethanolic solutions resulting in an ethanol concentration of 0.4 to 4% ethanol in the membrane suspension. While the rate of the oxy-hydrogen reaction was not impaired by ethanol, the rate of NADH-oxidation was reduced by 50% in *Pseudomonas facilis* and by 24% in strain *14 g* at a 4% ethanol concentration. Therefore, all data on the inhibitory effects on the NADH-oxidation have been corrected for the effect of ethanol; controls were run for the corresponding concentrations of ethanol without inhibitor. All measurements were made in parallel.

The experiments were restricted to the inhibitory action on the over-all electron transport pathway (NADH-oxidase and H_2-oxidase). The interpretation of the results was based on the assumption that the site of action of the inhibitors corresponds to those in other bacteria and mitochondria. In spite of this impairment, some drastic differences between NADH- and H_2-oxidation suggested partially separate electron transport systems in both strains.

Rotenone which is an inhibitor of flavoprotein action (Asano and Brodie, 1965; Imai *et al.*, 1967) slightly inhibited NADH- and H_2-oxidation of *P. facilis*, however, differentiated significantly between both oxidative pathways in strain *14 g* (Fig. 1). Thenoyltrifluoroacetone (TTFA) inhibited the reduction of non-heme iron protein in mito-

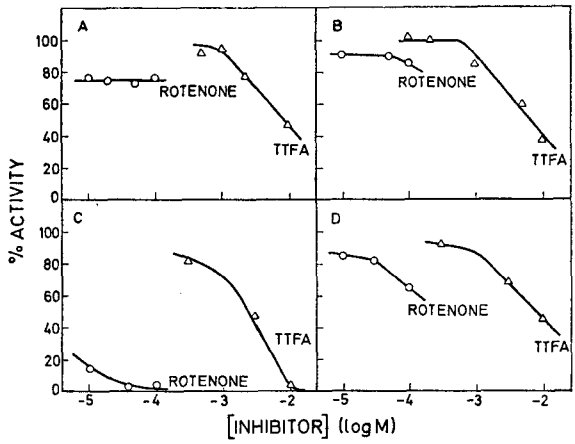

Fig. 1 A–D. Inhibition of the oxidation of NADH or H_2 by the membrane fraction of two hydrogen bacteria by rotenone and thenoyltrifluoroacetone. A NADH-oxidation by *Pseudomonas facilis*; B H_2-oxidation by *P. facilis*; C NADH-oxidation by strain *14 g*; D H_2-oxidation by strain *14 g*. Manometric measurements of gas uptake by membrane suspensions containing A 5.4 mg, B 0.64 mg, C 1.7 mg and D 1.52 mg membrane protein in 0.1 M K-phosphate buffer, pH 6.8 at a total liquid volume of 2.5 ml were carried out in the presence of either 5 mM NADH under 95% N_2 + 5% O_2 or without NADH under 95% H_2 + 5% O_2. Ethanolic solutions of the inhibitors were added. Data are given in percent of the ethanol containing, inhibitor free control. In the absence of inhibitors the following specific activities were measured: A: 7.9 nmole O_2/mg protein · min, B: 142 nmole O_2/mg protein · min, C: 36,7 nmole O_2/mg protein · min, D: 39.4 nmole O_2/mg protein · min

chondria between the flavoproteins of succinate oxidase and ubiquinone (Florkin and Stotz, 1966). The oxidation of both substrates was inhibited by TTFA, in both strains. A significant difference with respect to the degree of inhibition was only observed in the case of strain *14 g* (Fig. 1 C and D).

Electron transport between *cytochromes b* and *c* in bacterial systems was inhibited by antimycin A (Asano and Brodie, 1965; Bernofsky and Mills, 1966; Heinen, 1967), 2,3-dimercaptopropanol = BAL (Heinen, 1971) and 2-n-heptyl-4-hydroxyquinoline-N-oxide = HQNO (Asano and Brodie, 1965; Imai *et al.*, 1967; Lanyi, 1969). The effect of these inhibitors differed in both bacterial strains (Fig. 2).

a) Pseudomonas facilis: BAL did not influence the oxidation of NADH, however, impaired the oxidation of H_2. Antimycin A and HQNO inhibited both activities; Antimycin A mainly the oxidation of NADH, while HQNO exerted a greater effect on the oxidation of H_2. These differences were regarded to be significant and suggested separate pathways.

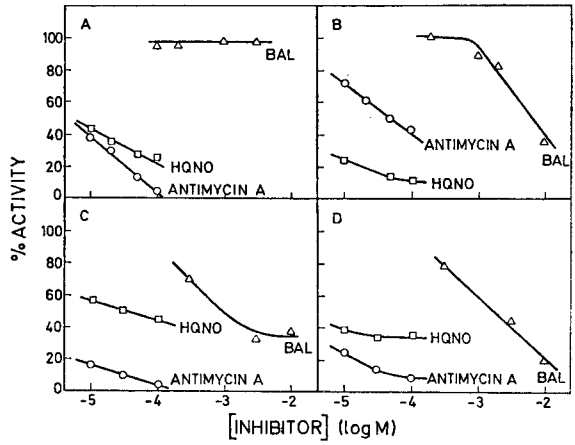

Fig. 2 A—D. Inhibition of the oxidation of NADH or H_2 by the membrane fractions of two hydrogen bacteria by antimycin A, BAL, and HQNO

b) Strain *14 g:* The differences of the effect of BAL, antimycin A, and HQNO on the oxidation of both substrates were rather small and did not suggest separate pathways.

The inhibition of both oxidation reactions by varying concentrations of potassium cyanide (10^{-5} to 10^{-3}) did not lend support to a differentiation between two pathways.

Discussion

Flavoproteins and *cytochromes a, b,* and *c* in the membrane fractions of *Pseudomonas facilis* and of strain *14 g* were reduced by hydrogen as well as NADH. These results obtained by difference spectrophotometry indicated that these redox carriers are involved in the electron transport chains of both substrates. By addition of the physiological substrates, the cytochromes became less reduced than in the presence of dithionite. An additive effect of both physiological substrates was not observed. These experiments, therefore, did not support the hypothesis that separate sets of redox carriers, each functioning with only hydrogen or NADH, are present.

However, rotenone and TTFA exerted inhibitory effects on the oxidation of NADH and H_2 by the membrane fraction of strain *14 g* which can be explained by the assumption of separate electron transport pathways at least to *cytochrome b.* On the basis of reverse effects of antimycin A and HQNO on the oxidation of H_2 and NADH and of the inhibition of only one of both reactions by BAL, one can postulate separate pathways at least to *cytochrome c* in *P. facilis.*

These results obtained for *P. facilis* are similar to those reported for *Hydrogenomonas eutropha H 16* (Pfitzner, 1969, 1972).

Although the conclusions are different from the paper on *H. eutropha* by Ishaque and Aleem (1970) the experiments are not in disaccordance with respect to the possible existence of a cytoplasmic NAD-dependent hydrogen dehydrogenase in this organism.

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