Arch. Microbiol. 112, 229–238 (1977)

Archives of
Microbiology

Localization and Stability of Hydrogenases from Aerobic Hydrogen Bacteria

KLAUS SCHNEIDER and HANS G. SCHLEGEL

Institut für Mikrobiologie der Gesellschaft für Strahlen- und Umweltforschung mbH München
und Institut für Mikrobiologie der Universität Göttingen, Grisebachstr. 8, D-3400 Göttingen, Federal Republic of Germany

Abstract. *Alcaligenes eutrophus* strains H 16, B 19, G 27 and N9A contained two different hydrogenases. One enzyme catalyzed the reduction of NAD by hydrogen and was strictly localized in the soluble cell fraction, while the second enzyme was found to be particulate and unable to react with NAD.

All other tested strains, *Alcaligenes paradoxus* SA 29, *Pseudomonas facilis*, *P. palleronii* RH 2, *Pseudomonas* sp. strain GA 3, *Paracoccus denitrificans*, *Aquaspirillum autotrophicum* SA 32, and *Corynebacterium autotrophicum* 14g and 7C contained only a single enzyme exclusively bound to membranes. This was established using fractional centrifugation, indicator enzyme systems, gentle methods of cell disintegration and discontinuous sucrose density gradient centrifugation. In cell-free extracts obtained by rough disruption (sonication) of cells, hydrogenase was associated to particles of different size and sedimentation velocity. A partial solubilization of hydrogenase caused by sonication was observed with *P. facilis*.

Without exception, the particulate hydrogenases were found (1) to be unable to reduce pyridine nucleotides, and (2) to reduce methylene blue at an extremely high activity. The eminent reaction rate of 34 µmoles H_2 oxidized per min and mg protein has been determined in particle suspensions of *Pseudomonas* sp. strain GA 3. All hydrogenases were stable during storage under hydrogen atmosphere, except the soluble enzyme from *A. eutrophus* H 16 which was shown to be more stable under aerobic conditions.

Key words: Hydrogen bacteria – Hydrogenase – Localization – Membrane bound enzymes – NAD reduction – Stability.

Depending upon the type of organism the function of hydrogenase in microbial metabolism is to oxidize or to produce molecular hydrogen. The aerobic hydrogen bacteria *Pseudomonas saccharophila* (Bone, 1960), *P. ruhlandii* (Vishniac and Trudinger, 1962) and *Alcaligenes eutrophus* (Wittenberger and Repaske, 1961; Eberhardt, 1966) apparently contain two types of hydrogenases, a soluble NAD reducing enzyme (hydrogen : NAD^+ oxidoreductase, E.C. 1.12.1.2) and a membrane bound enzyme, which is completely unable to react with NAD or NADP. Both enzymes are involved in the activation of H_2, but whereas the particulate hydrogenase is considered to be the first component of the electron transport chain, initiating the energy yielding process of hydrogen oxidation, the primary function of the soluble enzyme is thought to be the generation of reducing power (NADH) for CO_2-fixation.

In addition to *P. facilis* (Atkinson and McFadden, 1954; Ahrens, 1966), several strains of hydrogen bacteria have recently been described to contain a hydrogenase which is mainly associated with the particle fraction of cell-free extracts and does not react with pyridine nucleotides. These strains are *Paracoccus denitrificans* (Kühnemund, 1971), the yellow pigmented strains of *Corynebacterium autotrophicum* 12/60/x (Eberhardt, 1969), 14g (Schneider et al., 1973) and 7C (Tunail and Schlegel, 1974) and another coryneform but colourless bacterium, strain 11/x (Canevascini and Eberhardt, 1975). The failure of NAD(P) to function as H-acceptor indicated the absence of a soluble hydrogenase. However, the existing evidence for this statement is insufficient for two reasons: (1) As a rule, the cells have been disrupted by sonication and under aerobic conditions, which might have caused a complete enzyme inactivation. (2) A certain proportion of methylene blue reducing hydrogenase activity has always been retained

in the soluble cell fraction, and this activity was not definitively characterized.

These open questions prompted us to carry out more detailed investigations on the localization of hydrogenases from aerobic hydrogen bacteria.

MATERIALS AND METHODS

Organisms. The following strains of bacteria were used for investigations: *Alcaligenes eutrophus* (*Hydrogenomonas eutropha*) H 16 (ATCC 17699, DSM 428), *A. eutrophus* B 19 (DSM 515), *A. eutrophus* G 27 (DSM 516), *A. eutrophus* N9A (DSM 518), *Alcaligenes paradoxus* SA 29 (institute's culture collection), *Pseudomonas facilis* (ATCC 17695), *Pseudomonas palleronii* RH 2 (institute's culture collection), *Pseudomonas* sp. strain GA 3 recently isolated by Dr. G. Auling and similar to *P. flava*, *Paracoccus denitrificans* (DSM 413), *Corynebacterium autotrophicum* 14g (DSM 431), *C. autotrophicum* 7C (DSM 432) and *Aquaspirillum autotrophicum* SA 32 (DSM 732), a new hydrogen bacterium, recently isolated and taxonomically assigned (Aragno and Schlegel, 1977).

Cultivation and Harvesting of Cells. The bacteria were grown autotrophically in mineral medium according to Schlegel et al. (1961). 3 l of cell suspension were magnetically stirred at 30° C in 6 l flat-bottomed round flasks connected to the gas mixture of 80% H_2, 10% O_2 and 10% CO_2 contained in 10 l gasometer flasks. At the end of the exponential growth phase cells were centrifuged at 10000 × g for 30 min, washed twice in 0.067 M phosphate buffer, pH 7.0, resuspended in the same buffer to give a final density of 15–20 mg protein/ml and stored at −20° C.

Preparation of Cell-Free Extracts. The thawed cell suspensions were disintegrated by sonication as well as by the French press and by the treatment with lysozyme under various conditions.

Cells were sonicated (30 s/ml) using a 600 W sonic disintegrator (Schoeller and Co., Frankfurt). The temperature was kept below 10° C by cooling the vessel with −10° C ethanol.

Cell suspensions were passed once through a French pressure cell (Aminco, U.S.A.) at 80 kp/cm². Before use, the French press was cooled to 4° C.

Cells were centrifugated and resuspended in 50 mM Tris-HCl buffer (pH 8.0). After the addition of lysozyme (20 µg/mg wet weight), the suspension was incubated for 30 min at 37° C. Then DNase (20 µg/ml) was added, followed by further incubation for 15 min at the same temperature. The treatment of cell suspension with lysozyme was also carried out in the presence of EDTA (100 µg/mg wet weight) or polymyxine B (4 µg/mg wet weight) as described by Klemme (1972). The influence of pretreatment of cells with EDTA (Findley and Akagi, 1968) was also tested.

Unbroken cells and cell debris were removed by centrifugation at 10000 × g for 20 min. The supernatant was referred to as crude extract. The crude extract was fractionated into particulate and supernatant fractions by centrifugation at 100000 × g for 1 h and at 140000 × g for 3 h. The 100000 × g supernatant is designated as S1 fraction, the 140000 × g supernatant as S2 fraction. The particulate fractions P1 and P2 are represented by the 100000 × g and 140000 × g sediment, respectively.

Sucrose Gradient Centrifugation. Discontinuous sucrose gradients were prepared in 13 ml centrifuge tubes by layering sucrose solutions (1.5–2.5 ml) of different concentrations (10–70% w/v) one on top of the other. The sucrose was dissolved in 0.067 M phosphate buffer, pH 7.0. After carefully adding 1 ml cell-free extract on the top sucrose layer, the tubes were centrifuged at 26000 rpm for 16 h in a Beckmann Spinco L2 65B centrifuge. Particulate fractions and activity bands were localized by perforating the tubes, collecting 0.43 ml fractions and determining the enzyme activities.

Protein Determination. Protein was determined by the method of Beisenherz et al. (1953).

Enzyme Assays. Hydrogenase activity was measured manometrically by following the uptake of H_2 at 30° C. The reaction mixture in the Warburg flasks contained 0.067 mM phosphate buffer, pH 7.0, 5 mM methylene blue and the extract sample. The total volume of the reaction mixture was 2.2 ml. The shaking flasks were flushed for 15 min with O_2-free hydrogen and the reaction was started by tipping the solution of methylene blue from the sidearm into the main compartment.

Hydrogenase activity was also assayed by measuring the reduction of NAD spectrophotometrically in 1 cm cuvettes at 365 nm and at 30° C. The reaction mixture (3 ml) contained 0.067 M phosphate buffer (pH 8.0), 0.8 mM NAD and an appropriate amount of extract. All experiments were performed in anaerobic cuvettes closed with serum stoppers permitting the use of hypodermic needles to flush the cuvettes for 10 min continuously with a stream of O_2-free hydrogen before use. Buffer and NAD solution, both saturated with molecular hydrogen, were injected with a syringe. The reaction was started by injection of the extract sample. A unit of enzyme activity was defined as the reduction of 1 µmol NAD per min.

The activities of malate dehydrogenase and NADH oxidase were measured as described by Aggag and Schlegel (1973). Catalase activity was determined according to Bergmeyer (1955).

Chemicals. The chemicals used were obtained from: Boehringer (Mannheim), NAD, NADH, FMN, oxaloacetic acid, DNase, catalase; from Sigma Chemical Co. (St. Louis), polymyxine B; from Merck (Darmstadt), all other chemicals.

RESULTS

Hydrogenase Activity in Particulate and Soluble Fractions of Crude Extract

After sonication of autotrophically grown cells of the different strains of hydrogen bacteria, cell-free crude extracts were separated into different fractions by fractional centrifugation in order to investigate the distribution of hydrogenase activity. The efficiency of the fractionation procedure was controlled by determining NADH-oxidase as an indicator of a particle bound enzyme system and malate dehydrogenase as a soluble enzyme.

The existence of two different hydrogenase in *Alcaligenes eutrophus* were confirmed for the strains B 19, G 27, H 16 and N9A: A soluble, NAD reducing enzyme has been determined as well as a particle bound enzyme, which catalyzes the reduction of methylene blue but lacks the ability to reduce NAD. After high speed centrifugation of the crude extract at 100000 × g for 1 h, resulting in a particulate sediment and a supernatant fraction, the NAD reducing hydrogenase as well as the soluble malate dehydrogenase were strictly localized in the supernatant (Table 1). Further separation of periplasm and cytoplasm by applying lysozyme treatment in hypertonic

Table 1. Specific hydrogenase activities in cell-free extracts from the *Alcaligenes eutrophus* strains and the distribution of hydrogenase, malate dehydrogenase and NADH oxidase activity among the cell fractions. Crude extracts of cells disrupted by sonication were fractionated at 100000 × g for 1 h resulting in a sediment ("particles") and a supernatant ("soluble fraction")

Strain	Fraction	Specific hydrogenase activity (units/mg protein)		Relative activity (%)			
		NAD reduction	Methylene blue reduction	NAD reduction	Methylene blue reduction	NADH oxidase	Malate dehydrogenase
N9A	Crude extract	0.353	1.26	100	100	100	100
	Soluble fraction	0.436	1.04	88	60	44	99
	Particles	0	1.82	0	37	52	0.1
B 19	Crude extract	0.283	1.30	100	100	100	100
	Soluble fraction	0.285	1.18	61	56	34	99
	Particles	0	1.88	0	40	63	0.1
G 27	Crude extract	0.365	0.82	100	100	100	100
	Soluble fraction	0.472	0.64	77	41	27	95
	Particles	0	1.43	0	49	71	0.1
H 16	Crude extract	0.268	1.00	100	100	100	100
	Soluble fraction	0.380	0.54	87	29	42	99
	Particles	0.8	1.71	0.1	68	59	0.3

sucrose solution presented evidence that the soluble hydrogenase was not located in the periplasmic space as found with *Desulfovibrio gigas* (Bell et al., 1974), but was totally associated with the cytoplasmic cell fraction (Probst and Schlegel, 1976). The specific activity of NAD reduction determined in crude extracts amounted to about 0.350 – 0.50 units per mg protein. NADP was not reduced.

The methylene blue reducing activity was distributed to both fractions. 68% (H 16), 50% (G 27), and about 40% (B 19, N9A) were recovered in the particles, the residual activity was retained in the supernatants. This finding is to be explained by the following arguments: (1) Both the soluble and the particulate hydrogenase are able to react with methylene blue. (2) The sediment fractions were found to be free from soluble protein; the supernatants, however, were contaminated with small particles and membrane bound enzyme activities as demonstrated by the distribution of the indicator NADH oxidase (Table 1). The specific methylene blue reducing activity of the particle bound hydrogenase in the sediment fractions was 1.4 – 1.9 units per mg protein.

Surprisingly, in all other strains examined in this study, *Alcaligenes paradoxus* SA 29, *Pseudomonas facilis*, *P. palleronii* RH 2, *Pseudomonas* sp. strains GA 3, *Aquaspirillum autotrophicum* SA 32, *Paracoccus denitrificans* and *Corynebacterium autotrophicum* strains 14g and 7C, an enzyme able to reduce pyridine nucleotides was not encountered. Manifold variations in preparing the extracts and in conducting the enzyme assay were made with the aim to clarify the question, whether there is a hydrogenase which reduces NAD(P) in vivo. There might have been an enzyme, whose reaction conditions are not known or which is easily inactivated due to extreme sensitivity to oxygen or to rough cell disruption methods. Different buffer systems (phosphate, Tris-hydrochloride, triethanolamine) with varying concentrations and pH values were used; extract and NAD(P) concentration were increased to 4 mg protein per cuvette and 10 mM, respectively; several potential cofactors and electron carriers (divalent metal ions, NADH, methyl viologen, benzyl viologen, FMN, FAD, cytochrome *c*) were added and more gentle methods of cell breakage (French press and lysozyme treatment, repeated freezing and thawing of cell suspensions) were applied under aerobic as well as under strictly anaerobic conditions, using H_2-saturated buffer solutions made up from deaerated water and containing 25 mM dithionite. The results of all the experiments agreed: Crude extracts as well as soluble and particulate fractions are completely inactive to NAD(P).

A particle bound hydrogenase being of extremely high activity and able to reduce methylene blue was found in all strains. Under manometric assay conditions 2.5 (*C. autotrophicum*), 4 – 5 (*P. denitrificans*, *A. autotrophicum* SA 32), 10 – 11 (*P. facilis*, *P. palleronii* RH 2, *A. paradoxus* SA 29) and even 34 µmoles H_2 (*Pseudomonas* sp. strain GA 3) were oxidized per min and mg protein by the particulate fractions P1 (100000 × g sediments). These specific activities were up to 18 fold higher than those calculated for the particulate hydrogenases from the strains of *A. eutro-*

Table 2. Hydrogenase activity and its distribution among different fractions of sonic crude extract from hydrogen bacteria lacking an NAD reducing hydrogenase. Hydrogenase activity was measured manometrically by following the uptake of H_2 in the presence of methylene blue as H-acceptor

Organism	Fraction[a]	Specific hydrogenase activity (units/mg protein)	Relative activity (%)		
			Hydrogenase	NADH oxidase	Malate dehydrogenase
Paracoccus denitrificans	CE	1.42	100	100	100
	P1	5.13	61	75	2
	S1	0.73	33	25	96
	P2	3.05	90	93	30
	S2	0	0	6	65
Pseudomonas facilis	CE	3.86	100	100	100
	P1	9.66	81	67	0.2
	S1	0.88	12	30	99
	P2	5.85	93	87	17
	S2	0	0	13	80
Pseudomonas palleronii RH 2	CE	4.09	100	100	100
	P1	8.58	81	82	2
	S1	1.23	17	18	96
	P2	6.85	93	85	7
	S2	0	0	15	87
Pseudomonas sp. strain GA 3	CE	17.60	100	100	100
	P1	33.80	78	90	2
	S1	6.90	11	9	98
	P2	30.00	91	95	12
	S2	0.57	1	5	88
Alcaligenes paradoxus SA 29	CE	5.30	100	100	100
	P1	10.80	60	71	1
	S1	2.80	36	18	98
	P2	11.70	92	94	6
	S2	0.48	5	6	84
Corynebacterium autotrophicum 14g	CE	0.92	100	100	100
	P1	2.53	77	63	1
	S1	0.17	14	31	99
	P2	2.40	95	76	4
	S2	0	0	22	92
Corynebacterium autotrophicum 7C	CE	1.00	100	100	100
	P1	2.44	55	63	2
	S1	0.46	34	31	97
	P2	1.99	77	80	5
	S2	0.23	13	10	93
Aquaspirillum autotrophicum SA 32	CE	2.42	100	100	100
	P1	4.26	80	76	13
	S1	0.43	9	2	86
	P2	3.83	93	97	13
	S2	0.03	0.3	1	87

[a] Designation of fractions: CE = crude extract; P1 = 100000 × g sediment; P2 = 140000 × g sediment; S1 = 100000 × g supernatant; S2 = 140000 × g supernatant

phus. However, the methylene blue reducing activity was not associated with the P1 fractions only. In all strains a considerable portion of hydrogenase activity (9–36% of total activity) was localized in the supernatant S1 fractions (Table 2). The characterization of this activity was the aim of further studies. These served in answering the following questions: Is the hydrogenase activity found in supposedly soluble cell fractions associated with small residual particles, or has it to be assigned to a soluble hydrogenase unable to reduce NAD(P) but reacting with methylene blue, or has it to be considered as an artifact, originating from a partial solubilization of a membrane-bound enzyme?

After prolonged centrifugation of the crude extract at higher speed (140000 × g for 3 h) the hydrogenase activity in the soluble fractions S2 (140000 × g supernatants) was zero or less than 1%, with two exceptions: The S2-fractions of *A. paradoxus* SA 29 and *C. autotrophicum* 7C were found to contain 5 and 13% of the total hydrogenase activity, respectively. These values were consistent with the distribution pattern of the membrane bound NADH oxidases. On the other hand, in the S2 fractions of most of the strains superelevated NADH oxidase activities up to 13% (*C. autotrophicum* 14g) or even 22% (*P. facilis*) were observed. These activities, which were supposed to be due to the action of soluble flavoproteins, indicated that the membrane bound NADH oxidase was not a reliable indicator enzyme system. Furthermore, one has to consider that under the centrifugation conditions used, portions of even soluble proteins, corresponding to the molecular weight, were sedimenting (compare the distribution pattern of the malate dehydrogenases, Table 2). Thus, presented results provided first but not sufficient evidence that the methylene blue reducing activity, measured in "soluble" fractions, was also completely bound to particles.

Influence of Gentle Methods for Cell Disintegration on Yield and Distribution of Hydrogenase Activity

The primary intention to apply gentle methods for cell disintegration was to avoid the formation of artifacts such as destruction of membranes and solubilization of proteins.

Repeated freezing and thawing of cells turned out to be an excellent method to separate soluble and membrane bound proteins. Possibly due to the perforation of cell walls and cytoplasmic membranes by ice crystals, soluble proteins leaked through the cell envelopes, whereas particulate enzymes remained tightly bound to the cell membranes. Eight times freezing and thawing of cells from *C. autotrophicum* 14g, *P. denitrificans* and *Pseudomonas* sp. strain GA 3 resulted in the release of 14, 15 and even 64% of soluble protein, respectively, when referred to 100% malate dehydrogenase activity determined in sonic extract. Hydrogenase activity, however, was not detected at all even if extract concentrations up to a 10 fold increase were used. In a control test, the hydrogenase in soluble fractions of sonic extracts proved to be insensitive to freezing and thawing. The conclusion drawn from these results is that the hydrogenase is a 100% particulate enzyme.

Analogous results were obtained, when cells were treated with lysozyme (*A. autotrophicum* SA 32) or with lysozyme after pretreatment with EDTA (*A. paradoxus* SA 29) as described in "Materials and Methods". 25% (SA 32) and 50% (SA 29) yields of soluble protein contrasted to a hydrogenase activity that was absolutely zero. Lysozyme treatment probably caused no complete lysis but only cell damage, resulting in the release of only soluble and not particle bound enzymes into the medium.

Cells of *P. facilis* and *P. palleronii* RH 2 were found to be more sensitive to lysozyme. Treatment with lysozyme in the presence of EDTA or polymyxine B yielded a liberation of about 50% of the total cell protein and maximal activity of soluble enzymes. In these lysates a low level of hydrogenase activity was also found, which was, however, totally associated with particles recovered in the 100000 × g sediment.

C. autotrophicum 7C proved to be resistant against lysozyme treatment and against freezing and thawing. Extracts of cells passed once through a French pressure cell, exhibited a surprising distribution of hydrogenase activity: The percentages of hydrogenase activity in the "soluble" fractions S1 (53%) and S2 (40%) were even higher than those found in these fractions of sonicated cells (S1: 34%; S2: 13%). As this observation suggested the existence of a soluble hydrogenase, the analysis by sucrose gradient centrifugation should clarify this question.

Distribution of Hydrogenase in a Discontinuous Sucrose Gradient

Crude extract preparations of sonicated and French press treated cells of *C. autotrophicum* 7C were layered on top of a discontinuous sucrose gradient and fractionated by ultracentrifugation (26000 rpm for 16 h). The existence of a second soluble hydrogenase was unambiguously disproved by (1) the almost exact coincidence of activity profiles of hydrogenase and NADH oxidase (Fig. 1B), (2) the lack of enzyme activity in the supernatant, and (3) the controls having shown that the hydrogenase and the soluble reference

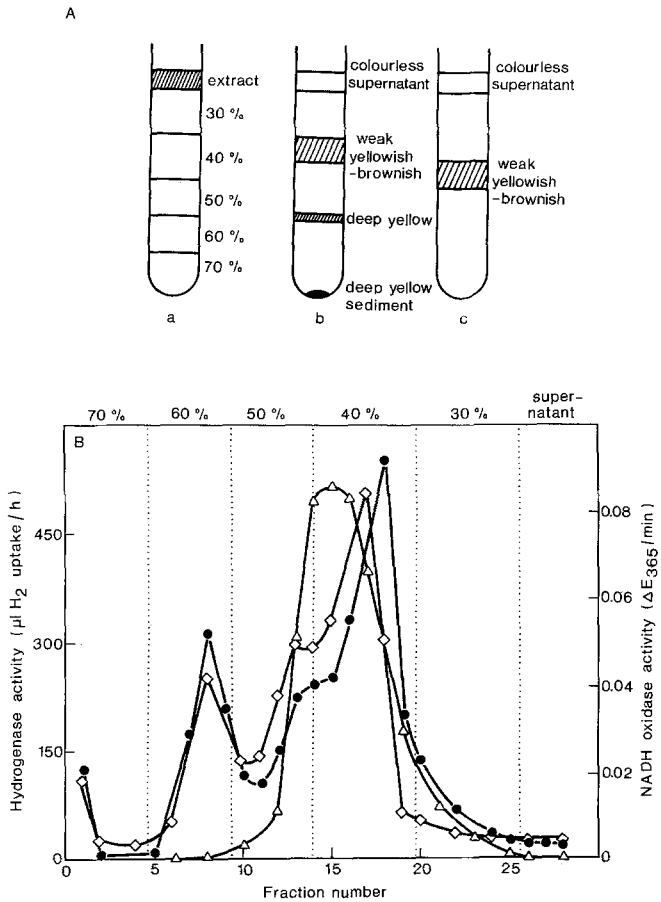

Fig. 1 A and B. Distribution pattern of hydrogenase activity and particles from *Corynebacterium autotrophicum* 7C in a discontinuous sucrose gradient. Discontinuous sucrose gradients were prepared as described in "Materials and Methods". Sucrose concentrations of 30–70% (w/v) were used. Volumes of the sucrose layers consisted of 2.5 ml (30 and 40% concentrated solutions) and 2 ml respectively (50–70% concentrated solutions). 1 ml crude extract was layered on top. After centrifugation at 26000 rpm for 16 h, the tubes were perforated and fractions of 0.43 ml were collected. (A) Banding of coloured particles; *a* discontinuous sucrose gradient as prepared before centrifugation; *b* ultrasonic extract, after centrifugation; *c* French press extract, after centrifugation. (B) Activity profiles of hydrogenase and NADH oxidase. ● Hydrogenase (sonic extract); △ hydrogenase (French press extract); ◇ NADH oxidase (sonic extract)

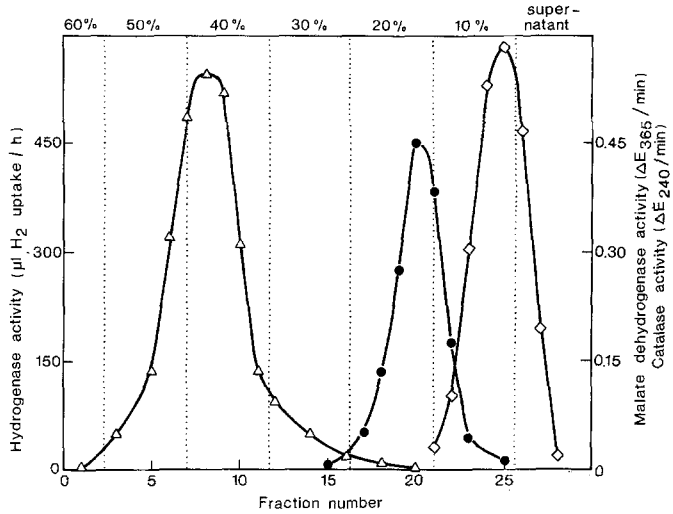

Fig. 2. Separation of particulate hydrogenase from *C. autotrophicum* 7C and soluble reference enzymes in a discontinuous sucrose gradient. Experimental conditions corresponded to those described in the legend to Figure 1 with the following modifications: 10–60% (w/v) sucrose solutions were used and the volume per layer was 2 ml; in the case of 10% sucrose, however, only 1 ml has been applied. Some crystals of catalase were dissolved in crude extract before centrifugation. △ Hydrogenase (French press extract); ● malate dehydrogenase; ◇ catalase

enzymes, malate dehydrogenase and catalase had been distinctly separated (Fig. 2).

The particles of sonic and French press extracts behaved differently during sucrose gradient centrifugation as observed at the distribution pattern of hydrogenase activity. When sonic extract was used, the hydrogenase activity profile revealed pronounced peaks in the 40% and 60% sucrose layer. Another maximum (shoulder) in the 50% sucrose layer and additional enzyme activity in the sediment fraction were also detected (Fig. 1 B). In contrast, the hydrogenase of the French press extract was bound to small particles of an apparently homogeneous size, localized in the 40% sucrose layer and marked distinctly as a strong symmetrical activity band (Figs. 1 B and 2). A sediment fraction and accumulations of particles in the region of 50–60% sucrose were absent. These results let us conclude that the larger particles recovered in the higher percent sucrose layers were supposed to be associations of membrane-cell wall fragments which are released as distinct fractions only after rough treatment of cells with sonics. This suggestion is supported by the postulation of Eberhardt (1971) that the bulk of the carotenoids of 12/60/x, another yellow pigmented strain of *Corynebacterium autotrophicum*, is located in the cell wall rather than in the cytoplasmic membrane. Indeed, an actual correlation has been observed between location of particles in the sucrose gradient, colour intensity and hydrogenase activity: The sedimented material and the particles forming a sharp band in the 60% sucrose layer exhibited a deep yellow colour and referred to this colour a relatively low activity, whereas the particles with the major activity were concentrated in the 40% sucrose layer as a very weak yellowish-brownish band (Fig. 1 A). Thus the colour intensity of particle fractions is considered to be an indicator for the degree of contamination of membranes with cell wall fragments.

The *P. facilis* hydrogenase is a membrane-bound enzyme exclusively. This was confirmed by sucrose gradient centrifugation. This method enabled us also to demonstrate that sonication of cells caused a partial

solubilization of the enzyme. When sonic extract was fractionated in the gradient, in addition to the main activity peak of hydrogenase at 50% sucrose, a small peak was recognizable in the 10–20% sucrose region, where soluble proteins were used to band, as indicated by catalase (Fig. 3). When French press extract was centrifuged under the same conditions, the small peak was absent and the main band of membrane bound hydrogenase became more pronounced.

Stability of Particulate and Soluble Hydrogenases

The stability properties of the particulate hydrogenases from all strains studied, proved to be remarkably similar. As demonstrated for the enzyme from *P. facilis* in Figure 4a, the particle suspensions were perfectly stable when stored at 4° C under molecular hydrogen. There was no loss of activity within 10 weeks. Reducing agents were not required. In the frozen state, a slow continuous decline of activity occurred amounting to 10–40% after 2 months. During storage under air at 4° C, a 45–65% decrease was observed within the first 2 weeks which was followed by a phase of nearly constant activity.

The stability of soluble hydrogenase from the *A. eutrophus* strains, except the H 16-enzyme, was higher in the presence of molecular hydrogen than in its absence, too. The loss of activity (up to 40% within 10 weeks at 4° C) was less when the enzymes were stored at −20° C (Fig. 4b). When kept under air, the soluble hydrogenases were shown to be rather labile. Storage at 4° C resulted in the complete loss of activity within a few days. Unexpectedly, the stability behaviour of the soluble hydrogenase from strain H 16 differed markedly from that of the other *A. eutrophus* strains. The enzyme was unstable in crude enzyme preparations but not sensitive to oxygen. H_2 caused no stabilization but rather an acceleration of enzyme inactivation (Fig. 4c). These observations are in accordance with the finding, that the purified enzyme, which is very stable in the oxidized state, is destabilized just by exposing to reducing conditions (Schneider and Schlegel, 1976).

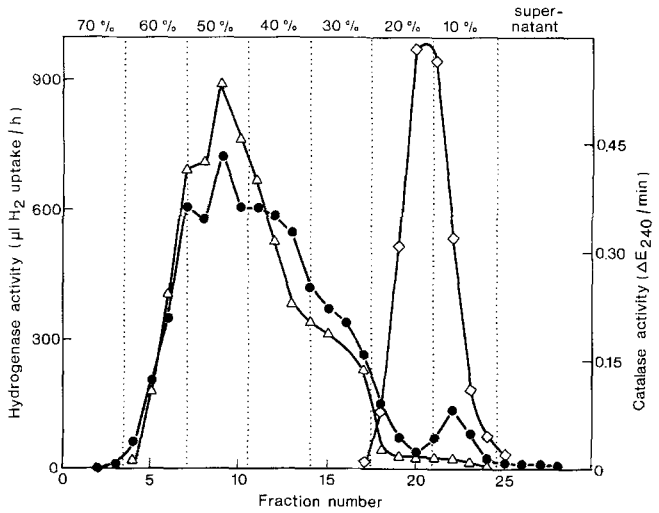

Fig. 3. Activity profile of the hydrogenase from *Pseudomonas facilis* in a discontinuous sucrose gradient. In a centrifuge tube 1.5 ml of seven 10–70% (w/v) concentrated sucrose solutions were layered one on top of another. Some crystals of catalase were added to the extract sample (1 ml) before application to the top sucrose layer. The gradient was centrifuged at 26000 rpm for 16 h and then eluted. Fractions of 0.43 ml were collected. ● Hydrogenase (sonic extract); △ hydrogenase (French press extract); ◇ catalase

DISCUSSION

Two types of hydrogenases have been described in aerobic hydrogen bacteria so far: a soluble hydrogen dehydrogenase able to reduce NAD (hydrogen : NAD^+ oxidoreductase, E.C. 1.12.1.2) and a membrane bound hydrogen oxidase of still unknown acceptor specificity and not reacting with pyridine nucleotides at all. Early investigations suggested that the majority of Knallgas bacteria would possess both types of enzymes. At first, Bone (1960), identified a soluble NAD reducing hydrogenase as well as a non-NAD-linked and particle associated activity in *Pseudomonas saccharophila*. Analogous results were obtained in subsequent studies of *P. ruhlandii* (Vishniac and Trudinger, 1962), *Alcaligenes eutrophus*, type strain (Wittenberger and Repaske, 1961) and *A. eutrophus* H 16 (Eberhardt, 1966). As the soluble hydrogenase serves the generation of reducing power

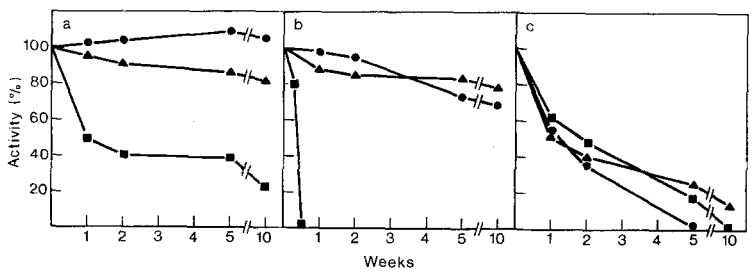

Fig. 4a–c. Stability of hydrogenases. Particulate hydrogenase from *P. facilis* (a) and soluble hydrogenases from *Alcaligenes eutrophus* B 19 (b) and H 16 (c) were stored as crude enzyme preparations at 4° C under H_2 (●), at −20° C under H_2 (▲) and at 4° C under air (■). Hydrogenase activity was determined at time intervals

Table 3. Distribution of soluble and particulate hydrogenase among aerobic hydrogen bacteria

Strain	Soluble hydrogenase	Particulate hydrogenase	References
Alcaligenes eutrophus, type strain and strains B 19, G 27, H 16, N9A	+	+	Wittenberger and Repaske (1961); Eberhardt (1966); present work
Alcaligenes paradoxus SA 29	−	+	present work
Pseudomonas saccharophila	+	+	Bone (1960)
Pseudomonas ruhlandii	+	+	Vishniac and Trudinger (1962)
Pseudomonas facilis	−	+	Atkinson and McFadden (1954); Ahrens (1966); present work
Pseudomonas palleronii RH 2	−	+	present work
Pseudomonas sp. strain GA 3	−	+	present work
Paracoccus denitrificans	−	+	Kühnemund (1971); present work
Aquaspirillum autotrophicum SA 32	−	+	present work; Aragno, unpublished
Corynebacterium autotrophicum strains 12/60/x 14g, 7C	−	+	Eberhardt (1969); Schneider et al. (1973); Tunail and Schlegel (1974), present work
Coryneform bacterium strain 11/x	−	+	Canevascini and Eberhardt (1975)
Nocardia opaca 1b	+	(−)	Aggag, unpublished

(NADH) needed for autotrophic CO_2 fixation, the occurrence of only a membrane bound hydrogenase in *P. facilis* (Atkinson and McFadden, 1954) was considered to be an exception. As manifested by the results of our comparative investigation and summarized in Table 3, it is just the opposite. The majority of species of the hydrogen bacteria contains only one single hydrogenase which is completely bound to membranes and unable to reduce NAD(P). Only a few species represented by *A. eutrophus*, *P. saccharophila* and *P. ruhlandii* possess in addition a soluble, NAD reducing hydrogenase.

Coordinated regulation of both enzymes in *A. eutrophus* H 16 and the failure to isolate stable mutants with only one single hydrogenase of appropriate activity (Schink, unpublished) raised the question, whether there are two independent different enzymes or only one enzyme with two different functions, exhibiting the phenomenon of allotopy (Racker, 1967). Significant differences between the purified enzymes with respect to serological and biochemical properties (Schink, unpublished) indicated, that there are indeed two different hydrogenases.

A hypothetical third and fourth type of hydrogenase, i.e. a particulate pyridine nucleotide reducing enzyme or a soluble enzyme not reacting with NAD(P) has not been encountered so far in any strain. The methylene blue reducing activity, measured in the "soluble" fraction of crude extracts was well characterized to be either associated to small, not sedimented particles or to be due to a hydrogenase partly solubilized during cell disruption by rough methods. This was demonstrated in the case of *P. facilis*.

An exceptional position among hydrogen bacteria is taken by *Nocardia opaca* 1b, which has been reported to catalyze hydrogenase dependent NAD reduction in both the particle and the soluble fraction of crude extract (Aggag and Schlegel, 1973). However, as the enzyme, localized in pellet material after fractional centrifugation, goes into solution just by repeated washings (Aggag, unpublished) it is strongly suggested, that *N. opaca* contains only a single and original soluble hydrogenase, which either is partly adsorbed to the membranes or forms aggregates with other proteins dissociating on further mechanical treatment.

Most hydrogenases of bacteria outside the hydrogen bacteria are also particulate or mainly associated with the cellular membrane fraction. However, only in a few cases (*Desulfotomaculum nigrificans*, *D. ruminis*, *Vibrio succinogenes*) has the hydrogenase been definitively described as a single particle bound enzyme (Akagi and Campbell, 1961; Buller and Akagi, 1964; Aspen and Wolin, 1966). In most cases, the residual activities found in the supposedly soluble cell fractions have not been clearly defined or the results were conflicting as in the case of the coli-aerogenes bacteria (Peck and Gest, 1954; Krasna and Rittenberg, 1957; Temperli et al., 1960; Ackrell et al., 1966). Purification and properties of a soluble (Yagi et al., 1968; Haschke and Campbell, 1971; LeGall et al., 1971) and a particulate hydrogenase (Yagi, 1970; Yagi et al., 1976) from *D. vulgaris* have been reported. Both enzymes are specific to cytochrome c_3 but seem to differ with respect to molecular and structural properties. However, soluble and particulate hydrogenase have been investigated from different strains, thus sufficient differentiation of both enzymes is also lacking for *P. vulgaris*. The involvement of more than one hydrogenase in hydrogen metabolism has also been assumed or not excluded for some photosynthetic bacteria

(Klemme, 1969; Gogotov et al., 1974). For *Azotobacter vinelandii* even the existence of two different particle bound enzymes has been discussed (Burns and Bulen, 1965). The existence of up to five "isoenzymes" of hydrogenase, differing in electrophoretic mobility has been postulated by Ackrell et al. (1966) for several bacteria species. Studies on the soluble hydrogenase from *Clostridium pasteurianum* (Nakos and Mortenson, 1971) and *A. eutrophus* H 16 (Schneider and Schlegel, 1976) however, revealed the "multiple forms" of hydrogenase as artifacts, which resulted from interactions of hydrogenase with other proteins and were formed only in crude enzyme preparations.

Single soluble hydrogenases have so far only been found in the strictly anaerobic bacteria *Micrococcus lactilyticus*, *Veillonella alcalescens*, *Butyribacterium rettgeri* (Ackrell et al., 1966), in the S-organism of *Methanobacillus omelianskii* (Reddy et al., 1972), in a number of clostridia (Ackrell et al., 1966; Kidman et al., 1969) and in *Desulfovibrio gigas* (Bell et al., 1974). The soluble enzyme from *D. gigas* has been reported to be located in the periplasmic space of the cell (Bell et al., 1974).

The physiological role of hydrogenase in aerobic hydrogen bacteria is twofold: (1) to generate reducing power, NAD(P)H, for CO_2-fixation; (2) to supply electrons for exergonic oxidative reactions. Thus a single enzyme fulfilling both functions (as found in *N. opaca* 1b) or the combination of two enzymes (as found in *A. eutrophus*), appear to be plausible. It is, therefore, surprising that the majority of species tested contain only a membrane bound hydrogenase which does not reduce NAD(P). The question which remains, is, how the organisms gain their NAD(P)H required for reductive, biosynthetic reactions. As the natural electron acceptor of membrane bound hydrogenase apparently is a higher potential component of the respiratory chain, it is suggested, that a reverse electron flow operates and that NAD is reduced indirectly through an energy dependent reaction.

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Received October 21, 1976