PROPERTIES OF HYDROGENASE FROM *MEGASPHAERA ELSDENII*

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PROPERTIES OF HYDROGENASE FROM *MEGASPHAERA ELSDENII*

Proefschrift

ter verkrijging van de graad
van doctor in de Landbouwwetenschappen,
op gezag van de Rector Magnificus,
dr. H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op woensdag 17 september 1980
des namiddags te vier uur in de Aula
van de Landbouwhogeschool te Wageningen

Omslagontwerp: E.J. van Dijk

STELLINGEN

1

Hageman en Burris hebben bij het bepalen van de oxidatiesnelheid van ditioniet door *Azotobacter vinelandii* dinitrogenase in aanwezigheid van het homologe flavodoxine geen rekening gehouden met de spectrale veranderingen van het flavodoxine gedurende het verloop van de reactie.

-Hageman, R.V. en Burris, R.H. (1978) Biochemistry, *17*, 4117-4125.

2

Het *in vitro* replicase systeem van poliovirus zoals beschreven door Dasgupta *et al.* lijkt, op grond van de aan VPg toegeschreven rol bij de initiatie van de RNA replicatie, voorlopig incompleet te zijn.

-Dasgupta, A., Zabel, P. en Baltimore, D. (1980) Cell, *19*, 423-429.

-Dasgupta, A., Baron, M.H. en Baltimore, D. (1979) Proc. Natl. Acad. Sci. U.S.A. *76*, 2679-2683.

-Petterson, R.F., Ambos, V. en Baltimore, D. (1978) J. Virol. *27*, 357-365.

-Nomoto, A., Detjen, B., Pozzatti, R. en Wimmer, E. (1977) Nature, *268*, 208-213.

3

Een multiple choice examen vergemakkelijkt het leven van de examinator, maar devalueert de mogelijkheid tot schriftelijke expressie van de examinandus.

4

De relatief lage serumcholesterol concentraties bij vegetariërs kan niet worden verklaard door de consumptie van alleen plantaardig eiwit, daar voedingseiwit geen effect op deze concentratie heeft bij normocholesteremische proefpersonen.

-Burslem, J., Schonfeld, G. en Howald, M.A. (1978) Metabolism, *27*, 711-719.

-van Raaij, J.M.A. (1979) Lancet II, 958.

5

De neiging die bij vele onderzoekers bestaat om gemeten waarden te optimaliseren is te veroordelen.

-Reed, L.J., Pettit, F.H. en Eley, M.H. (1975) Proc. Natl. Acad. Sci. U.S.A. *72*, 3068-3072.

-Hinkle, P.C. en Yu, M.L. (1979) J. Biol. Chem. *254*, 2450-2455.

-Emptage, M.H., Kent, T.A. en Huynk, B.H. (1980) J. Biol. Chem. *255*, 1793-1796.

6

De door Yagi *et al.* waargenomen veranderingen in intensiteit van ^{13}C-NMR spectra van verrijkt FMN onder verschillende omstandigheden kunnen worden verklaard door NOE (Nuclear Overhauser Enhancement) en spin-rooster relaxatie verschillen.

-Yagi, K., Ohishi, M., Takai, A. (1976) Flavins and Flavoproteins (Singer, T.P. ed) pp. 775-781, Elsevier, Amsterdam.

7

Belangstellenden moeten in de gelegenheid worden gesteld grond te huren van de gemeente voor het aanleggen van volkstuinen.

8

Zonder het zich te realiseren, laten Andersen en von Meyenburg zien, dat *Escherichia coli* een aktief, energie-afhankelijk transhydrogenase bevat.

-Andersen, K.B. en von Meyenburg, K. (1977) J. Biol. Chem. *252*, 4151-4156.

9

De veronderstelling dat er een correlatie bestaat tussen de midpoint potentiaal van de electron carrier en de waterstofproduktie aktiviteit van het hydrogenase van *Rhodospirillum rubrum* dient zeer sceptisch te worden bezien.

-Adams, M.W.W. en Hall, D.O. (1979) Biochem. J. *183*, 11-22.

10

De lichamelijke conditie van wetenschappers is een academische vraag.

Aan Yvonne, Fulko en Nienke
Aan mijn ouders

VOORWOORD

Bij het tot stand komen van dit proefschrift ben ik aan velen dank verschuldigd. Met name wil ik noemen:

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CONTENTS

LIST OF ABBREVIATIONS

A 580	absorbance at 580 nm
albumin	bovine serum albumin
BV^{2+}	oxidized benzyl viologen
Cl_2Ind	2,6-dichloroindophenol
EDTA	ethylene diaminetetra acetate
E_h	potential of an oxidation-reduction system with reference to the hydrogen standard
$E_{m,7}$	E_h at the midpoint of a symmetrical titration curve at pH 7
E_o	E_h when S_o/S_r = 1 and pH = 0
EPR	electron paramagnetic resonance
$[E]_t$	total enzyme concentration in $mg.ml^{-1}$
Fd_{ox}	oxidized ferredoxin
Fd_{red}	reduced ferredoxin
Fl	oxidized flavodoxin
FlH_2^-	unprotonated species of flavodoxin hydroquinone
FlH_3	protonated species of flavodoxin hydroquinone
FMN	riboflavin 5'-phosphate
h	Hill coefficient
Hipip	high-potential iron-sulphur protein
K	equilibrium constant
k	rate constant
K_m	Michaelis constant
Me_2SO	dimethyl sulphoxide
M_r	relative molecular mass
MV^{2+}	oxidized methyl viologen
MV^+	methyl viologen semiquinone
$(MV)_2^{2+}$	methyl viologen semiquinone dimer

$[S]_{0.5}$	substrate concentration at which the rate of reaction $v = \frac{1}{2}V$
S_o	sum of the molar concentrations of oxidant
S_r	sum of the molar concentrations of reductant
V	rate of reaction at infinite concentration of substrate
v	rate of reaction

1. GENERAL INTRODUCTION

In recent years mankind became aware of the impending shortage of fossil fuels. As a consequence of this 'energy' crisis the search for alternative fuel sources has been greatly accelerated. Although many new concepts have been proposed, solar energy has now been duly recognized as the largest and ultimate non-fossil, non-nuclear energy resource. However, firstly, the solar energy conversion efficiency has always been relatively low [1] and, secondly, mankind has not yet learned to harness solar energy on a scale commensurate with his ever-increasing energy demands. On the other hand, the magnitude of the solar radiation that reaches the earth surface is much greater than any of the foreseeble needs, so that harnessing solar energy represents an inviting technical target. One of the possibilities for solar energy conversion is the biological and biochemical photo-production of molecular hydrogen[a)]. The capability of many photosynthetic microorganisms to produce hydrogen gas has been recognized for many years [2]. This process is directly or indirectly linked to the light-dependent photosynthetic pathway. However, since the efficiency of this conversion process is relatively low, together with the fact that hydrogen production is often sensitive to oxygen (a product of photosynthesis) studies on the possible application of this pathway have been precluded in applied research. The biological system used for solar energy conversion to produce hydrogen consists of a photosystem, either natural (chloroplasts) or artificial (photo-receptor plus electron donor), an appropriate electron transferring species (natural; flavodoxin, ferredoxin, cytochromes, or artificial; low potential dyes) and the enzyme hydrogenase. The photosystem enables the formation of electrons with a sufficiently low redox potential, which are transferred to hydrogenase, to catalyze the production of hydrogen gas. Since hydrogenase is an integral component of such a system, knowledge of its structural, physico-chemical and catalytic properties is essential for its efficient coupling to the photosystem. Biological photoproduction of hydrogen, as opposed to thermo-chemical and physical methods, and hydrogen as fuel have several advantages:

a) Molecular hydrogen (= gaseous hydrogen= dihydrogen = H_2) will be designated as hydrogen in the text.

1) The biological systems operate at physiological temperatures which are generally much lower than required for chemical or physical production of hydrogen.
2) The only major input in the system would be solar energy and a hydrogen donor, probably (salt) water if chloroplasts are used, however, another source of electron donor might be necessary using an artificial photo-receptor.
3) The production of hydrogen would not involve the formation of pollutants, as is the case with fossil fuel refineries.
4) The fuel developed, hydrogen gas, would be clear burning, provided that pure oxygen is used (nitrous oxides are formed if air is used) with a high energy yield (ΔG^{O} = -474.5 kJ/reaction).
5) The hydrogen produced can be used as a chemical feedstock in the production of ammonia, methanol, refined petroleum fuels, hydrogenated vegetable and animal oils and other chemicals.

However, besides advantages photoproduction of hydrogen also has problems:
1) Inactivation of the (reduced) hydrogenase in the presence of oxygen if chloroplasts are used as photoreceptor.
2) Inherent instability of the photosynthetic apparatus and hydrogenase and also biodegradation.
3) Only part of the light can be used by the photosynthetic apparatus.

Hydrogen formation from organic matter might also be one of the possibilities to produce hydrogen. In this case biomass can be considered to represent the end product of solar energy conversion.

1.1. HYDROGENASE

As has been pointed out above, knowledge of the structure, physico-chemical and kinetic properties of hydrogenase is essential for its efficient coupling to the photosystem. This thesis deals with the properties of hydrogenase from *Megasphaera elsdenii*.
Hydrogenases are defined as:" Enzymes catalyzing the reversible activation of hydrogen", according to:

$$2e + 2H^{+} \rightleftarrows H_2 \qquad (1)$$

However, since the discovery of the so-called unidirectional hydrogen-oxydizing hydrogenase [3], a better definition for hydrogenases would be: "Enzymes catalyzing the activation of dihydrogen". This definition excludes nitrogenase as a hydrogenase since nitrogenase is only capable of producing hydrogen in an ATP-

dependent reaction [4].

1.1.1. Historical background

In 1887 Hoppe-Seyler [5] observed production of equal amounts of H_2 and CO_2 from a calcium solution inocculated with river mud, and he attributed this to the presence of bacteria. Pakes and Jollyman [6] showed that pure cultures of the colon-typhoid bacteria produced mixtures of CO_2 and H_2 from glucose or formic acid. In 1931 Stephenson and Stickland [7] demonstrated the existence of the enzyme hydrogenase in *Escherichia coli*, which was able to reduce methylene blue with H_2.

Besides the wide spread occurrence of hydrogenases [8,9] the purification and characterization was often hampered by the association of the enzyme with cell membranes, or its oxygen sensitivity. About four decades after the discovery of hydrogenase, reasonable purified preparations were reported of the enzymes from *Clostridium pasteurianum* [10] and *Desulfovibrio vulgaris* [11-13]. Nowadays many purified preparations of hydrogenase from a wide range of microorganisms have been described, including from obligate anaerobic [3,14-24], from anaerobic phototrophic [25-29], from facultative anaerobic [30,31], from aerobic phototrophic [32] and from aerobic chemoautotrophic [33-37] bacteria.

1.1.2. Hydrogen evolution in heterotrophs

In the production of hydrogen by strict anaerobes, such as *M.elsdenii* and *C.pasteurianum* and facultative anaerobes, for example *E.coli* and *Proteus mirabilis*, pyruvate plays a key role. Several important enzymes and electron carrier(s) function in the dissimilatory breakdown of pyruvate, resulting in the reduction of protons to form hydrogen. Figure 1 gives a general scheme of the proposed electron flow to produce hydrogen by the obligate anaerobes and facultative anaerobes, the latter growing under anaerobic conditions.

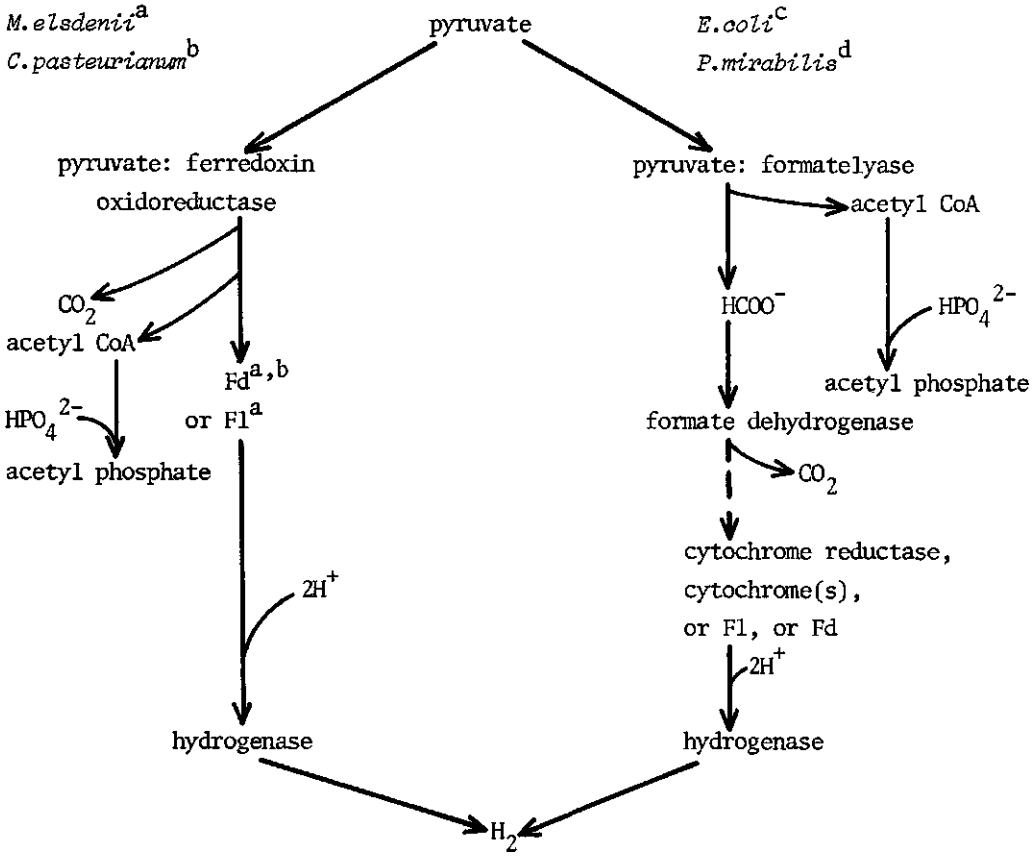

Figure 1. *General scheme for the production of hydrogen gas from the anaerobic breakdown of pyruvate by the obligate anaerobes* M.elsdenii *and* C.pasteurianum *and by the facultative anaerobes* E.coli *and* P.mirabilis.
(⟶) electron flow; (⇢) postulated electron flow; a, references for *M. elsdenii* [38-40]; b, references for *C. pasteurianum*, see [8,9] and references therein; c, references for *E. coli*, see [8,9,31] and references therein; d, references for *P. mirabilis*, see [30] and references therein; Fd, ferredoxin, Fl, flavodoxin.

In the obligate anaerobes pyruvate is dissimilated by pyruvate: ferredoxin oxidoreductase:

$$CH_3.CO.COO^- + HSCoA + Fd_{ox}/2FlH_2^{\cdot} \rightarrow CH_3.CO.SCoA + Fd_{red}/2FlH_3 \qquad (2)$$

$E_{m,7} = -510$ mV [41].

The thioester energy bond of acetyl CoA is conserved as ATP through the intermediate acetyl phosphate by phosphotransacetylase (reaction (3)) and the enzyme acetate kinase (reaction (4)):

$$CH_3.CO.SCoA + HPO_4^{2-} \rightarrow CH_3.CO.PO_4^{2-} + HSCoA \quad (3)$$

$$CH_3.CO.PO_4^{2-} + ADP^{3-} \rightarrow CH_3.COO^- + ATP^{4-} \quad (4)$$

The electrons, in the form of reduced ferredoxin or flavodoxin hydroquinone are disposed of via hydrogenase:

$$2H^+ + Fd_{red}/2FlH_3 \rightarrow H_2 + Fd_{ox}/2FlH_2^{\cdot} \quad (5)$$

$E_{m,7} = -420$ mV [41].

Reaction (5) depicts the "valve" function of hydrogenase; to release excess reducing power in the form of hydrogen in order to obtain extra energy from substrate level phosphorylation.

In the facultative anaerobes, under anaerobic conditions, pyruvate is broken down by pyruvate: formatelyase:

$$CH_3.CO.COO^- + HSCoA \rightarrow CH_3.CO.SCoA + HCOO^- \quad (6)$$

The formate decarboxylation reaction is catalyzed by the formate hydrogenlyase complex, which consists of formate dehydrogenase, probably at least two distinct electron transferring proteins, and hydrogenase.

$$H^+ + HCOO^- \rightarrow \ldots\ldots \rightarrow \ldots\ldots \rightarrow CO_2 + H_2 \quad (7)$$

$E_{m,7} = -420$ mV [41].

The acetyl CoA moiety is also conserved as ATP through the reactions (3) and (4).

For the facultative anaerobes hydrogenase also serves as "valve" to dispose of excess reducing equivalents in order to obtain energy from susbstrate level phosphorylation. However, depending on the anaerobic growth conditions electrons from the oxidation of formate are either transferred to a terminal electron

acceptor by way of the respiratory chain (for example nitrate as terminal electron acceptor (see refs. [8,9,30,31] and references therein)) or put into the hydrogenlyase system to produce CO_2 and H_2.

1.1.3. A survey on the types of hydrogenases

Many microorganisms of different physiology and taxonomy contain the enzyme hydrogenase [8,9], depicting the metabolic versatility of microorganisms as well as the key role for hydrogenase in energy metabolism. Recent reviews about the physiological role of hydrogenase in microbial energy metabolism are given by Mortenson and Chen [8], Zajic *et al.* [9] and by Schlegel and Schneider [42]. From these reviews it becomes clear that, with exception of the obligate anaerobic fermentative bacteria, the role of hydrogenase in energy metabolism is strongly dependent on the growth conditions applied to the particular microorganism, making generalizations difficult if not impossible.

The most relevant physico-chemical and kinetic properties of purified hydrogenase preparations so far described are summarized in Table 1. This table clearly shows that "grouping" of hydrogenases based on the above mentioned characteristics meets the same problem as ascribing a uniform role to hydrogenase in energy metabolism. However, as is the case for the role of hydrogenase in energy metabolism of the obligate anaerobic fermentative bacteria (see Table 1) striking similarities are observed between the hydrogenases of *M.elsdenii* [23,24] and *C.pasteurianum* [14-19] with respect to physico-chemical and catalytic properties. The hydrogenase from *Desulfovibrio vulgaris* (Hildenborough), as isolated by van der Westen *et al.* [21], shows besides similarities (molecular weight, Fe-S content, catalytic activity with methyl viologen semiquinone) also great differences (location, natural electron donor, EPR spectra) and therefore has another E.C. number (hydrogen: cytochrome c_3 oxidoreductase; EC 1.12.2.1) as compared to the EC number of the hydrogenases from *M.elsdenii* and *C.pasteurianum* (hydrogen: ferredoxin oxidoreductase; EC 1.18.3.1). Although belonging to the same genus the hydrogenases of *D.vulgaris* (Miyazaki) [20] and *D.gigas* [22] have quite different kinetic and physico-chemical properties (lower activities and non-identical subunit structure) as compared with the hydrogenase of *D.vulgaris* (Hildenborough) [21].

Some similarity in properties is apparent between the hydrogenases of the anaerobic phototrophic bacteria (see Table 1). These bacteria contain a hydrogenase which is/tends to be particulate; its natural electron donor might be a fer-

Table I *Catalytic, structural and physico-chemical properties of various hydrogenases*

++ very oxygen sensitive; + oxygen sensitive; - oxygen insensitive; -- very oxygen insensitive

Micro-organism	*Megasphaera elsdenii*	*Clostridium pasteurianum*	*Desulfovibrio vulgaris* (Hildenborough)	*Desulfovibrio vulgaris* (Miyazaki)	*Desulfovibrio gigas*	*Chromatium*	*Chromatium vinosum*	*Rhodospirillum rubrum*	*Thiocapsa roseopersicina*	*Proteus mirabilis*	*Escherichia coli*	*Alcaligenes eutrophus*	
Physiology	obligately anaerobic fermentative		obligately anaerobic, with electron transport phosphorylation			anaerobic	phototrophic			facultatively anaerobic		aerobic chemoautotrophic	
Localization	cytoplasm?	cytoplasm	periplasm	membranes	periplasm	membranes	soluble	membranes	soluble and partic. fraction	membranes	membranes	cytoplasm	membranes
Natural electron donor/acceptor	flavodoxin ferredoxin	ferredoxin	cytochrome c_3	cytochrome c_3	cytochrome c_3	unknown	unknown	ferredoxin I,II? cyt "c_3"?	cytochrome cyt "c_3"	unknown	unknown	NAD^+	unknown
Molecular weight	50.000	60.500	50.000	89.000	89.500	98.000	55.000	66.000	68.000	205.000	113.000	200.000	98.000
Subunits	1 x 50.000	1 x 60.500	1 x 50.000	1 x 59.000 1 x 28.000	1 x 62.000 1 x 26.000	2 x 50.000	1 x 55.000	1 x 66.000	1 x 25.000 1 x 47.000	2 x 63.000 2 x 33.000	2 x 56.000	1 x 68.000 1 x 60.000 2 x 29.000	1 x 67.000 1 x 31.000
Extinction coefficient of oxidized form ($mM^{-1}cm^{-1}$, 400 nm)	46	36	46	47	46.5	14				106	49	60 (A_{420})	30 (A_{408})
Iron (g. atom/mol Acid-labile Sulphide (g.atom/mol Flavin/mol	12 12 --	12 12 --	12 12 --	8 8 --	12 12 --	4 4 --	4 4 --	4 4 --	4 4 --	24 24 --	12 12 --	12 12 2	6 6 --
Structure of the Iron-Sulphur centres	3 x (4Fe-4S)	3 x (4Fe-4S)	3 x (4Fe-4S)		3 x (4Fe-4S)	1 x (4Fe-4S)	1 x (4Fe-4S)	1 x (4Fe-4S)				2 x (4Fe-4S) 2 x (2Fe-2S)	
EPR signals: oxidized	2.101, 2.052 2.005	2.099, 2.046 2.005	2.029-1.995			2.019, 2.003	1.96, 1.96, 1.98, 2.003, 2.018, 2.03	2.023, 2.004		2.025, 2.018		2.02	
reduced	2.087-1.89	2.079-1.89	2.055-1.893			2.2, 2.125, 2.06	no signal	no signal		no signal		2.04, 1.95[a] 2.04 - 1.86[b]	
Oxygen sensitivity	++	++	--			--	--	--	--	--	+	--	--
V (μmol H_2 produced.min^{-1}.mg^{-1})	7000, pH 8	4000	10.400	90	91			33.3			13.6	48	
MV^+ as electron donor	140.000, pH 5.5	pH 8.0	pH 8.0	pH 7.0	pH 7.6			pH 7.0			pH 7.0	pH	
References	23,24 this thesis	14-18	21	20	22	25	29	26,43	28	30	31	33-35	36

a. Observed at 50-77 K
b. Observed at <30 K

redoxin and/or a low potential cytochrome. The enzymes of the anaerobic phototrophic bacteria contain one 4Fe-4S cluster which, in the oxidized state, shows an EPR spectrum of the so-called Hipip type (high-potential iron-sulphur protein). The hydrogen production activity of these enzymes is, compared to the activity of the enzymes from *M.elsdenii* [23], *C.pasteurianum* [14] and *D.vulgaris* (Hildenborough) [21], very low (0.3 - 0.8%).

A hydrogenase with very outstanding properties as compared to the other enzymes of Table 1 is the soluble hydrogenase from *Alcaligenes eutrophus* [33-35]. Besides its complex subunit structure and its lack of sensitivity towards oxygen, it contains besides 12Fe and 12S, which are arranged in two 2Fe-2S and two 4Fe-4S clusters [35], two additional FMN molecules [34]. These FMN molecules probably enable this enzyme to reduce NAD^+. However, despite the fact that this enzyme contains 12Fe and 12S its hydrogen production activity is also rather low; this is also the case for the 12Fe-12S and 24Fe-24S containing enzymes from respectively *E.coli* [31] and *P.mirabilis* [30].

1.2. OUTLINE OF THIS THESIS

This thesis deals with the anaerobic purification and properties of the hydrogenase (EC 1.18.3.1) from the obligate anaerobic rumen bacterium *Megasphaera elsdenii*. Since this enzyme is a redox enzyme catalyzing the overall reaction:

$$2e + 2H^+ \rightleftarrows H_2 \qquad (1)$$

the effects of the electron concentration (in the form of an appropriate electron donor) and proton concentration, in relation to the redox potential on the hydrogen production activity, as well as the hydrogen oxidation activity were studied, resulting in a mechanism for the action of this enzyme. Furthermore the effects of oxygen, salts, Me_2SO and ethylene glycol on the activity were studied. The different chapters of this thesis describe the following aspects:

2. Purification and characterization of hydrogenase from *M.elsdenii*, with respect to kinetics with flavodoxin hydroquinone, reduced ferredoxin and methyl viologen semiquinone.
3. Effects of oxygen, salts, Me_2SO and ethylene glycol, and storage on the hydrogenase activity, as well as EPR spectroscopy of hydrogenase from *M.elsdenii*.
4. An analysis of the theoretical aspects of product formation in a series

coupled redox reactions, with special reference to hydrogenase.

5. The effects of pH and redox potential on the hydrogen production activity of hydrogenase from *M.elsdenii*.
6. Summary.
7. Samenvatting.

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2. PURIFICATION AND PROPERTIES OF HYDROGENASE FROM *MEGASPHAERA ELSDENII*

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A hydrogenase has been purified to homogeneity from the soluble fraction of the rumen bacterium *Megasphaera elsdenii*, the overall purification is 200 times with a yield of 14%. The pure enzyme consists of a single polypeptide chain with $M_r \approx 50000$ which contains 12 atoms of non-haem iron and 12 atoms of acid-labile sulphide. The enzyme is rapidly inactivated by O_2 and it is therefore purified under nitrogen and in the presence of sodium dithionite. The optical spectrum of the enzyme, after removal of the dithionite with air, shows a peak at 275 nm ($\varepsilon_{275\,nm} = 143\ mM^{-1}\ cm^{-1}$) and a shoulder between 350 nm and 400 nm ($\varepsilon_{400\,nm} = 46\ mM^{-1}\ cm^{-1}$).

The enzyme catalyses hydrogen production from sodium dithionite at a low rate. The rate is greatly enhanced by addition of the electron donors flavodoxin, ferredoxin and methyl viologen. The kinetic data with these three electron donors suggest co-operativity, but no indication of self-association of the enzyme was obtained. Sodium chloride enhances the rate of hydrogen production with methyl viologen semiquinone and changes the kinetic behaviour of the enzyme with this electron donor, but causes inhibition of the reactions mediated by ferredoxin and flavodoxin.

Two kinetic models were developed which are consistent with the kinetic data of the three electron donors tested. The apparent co-operativity for the hydrogen production can be fitted with the mathematical form of those models. The identical kinetic behaviour of the hydrogenase with the one-electron donors flavodoxin and methyl viologen semiquinone monomer and the two-electron donor ferredoxin indicates that the hydrogenase accepts two electrons in two separate, independent steps and further indicates that the two (4Fe-4S) clusters of the donor ferredoxin are independent. The interpretation of the kinetic data with methyl viologen semiquinone is complicated by the fact that the semiquinone dimerises, and that the formation of the dimer is enhanced by salt. Taking into account the association of this donor, the activity of the enzyme with methyl viologen semiquinone can be described by the sum of the activities of the enzyme with methyl viologen monomer and methyl viologen dimer.

The enzyme catalyses the oxidation of hydrogen gas with methyl and benzyl viologen as electron acceptors to their semiquinone forms; both electron acceptors show Michaelis-Menten kinetics. The hydrogen oxidation activity with both electron acceptors is stimulated by addition of sodium chloride. The kinetic data of the oxidation of hydrogen with the two-electron acceptors used are consistent with the proposed models, if it is assumed that the pathway followed is compulsory.

At this moment no choice can be made between the models proposed.

Purified preparations of hydrogenase have been described from several microbial sources, including strictly anaerobic bacteria [1–12]. The preparation that has been studied in most detail was obtained from the anaerobe *Clostridium pasteurianum* by Chen and Mortenson [1,13–15]. This enzyme consists of a single polypeptide chain with $M_r \approx 60000$ which contains 12 atoms of non-haem iron and an equivalent amount of acid-labile sulphide. An enzyme preparation with similar molecular properties was isolated more recently from a second anaerobe, the sulphate-reducing bacterium *Desulfovibrio vulgaris* (strain Hildenborough) [6]. Both preparations show high catalytic activity with dithionite-reduced methyl viologen. The desulfovibrio enzyme differs from the clostridial enzyme, however, in using cytochrome c_3 rather than ferredoxin as the natural electron donor/acceptor, and in further contrast to the clostridial enzyme, it is stable in air. Additional preparations of purified hydrogenase have been reported from these two bacteria [2–4], but the molecular and/or catalytic properties of the preparations differed markedly

Enzymes (IUB Recommendations 1978). Hydrogenase or hydrogen:ferredoxin oxidoreductase (EC 1.18.3.1).

from those described by Chen and Mortenson [1, 13–15] and Van der Westen et al. [6]; the differences have not yet been explained. Preparations of hydrogenase have been reported from the Miyazaki strain of *D. vulgaris* [5], and also from *Desulfovibrio gigas* [7], but their specific activities were low and they differed in other respects from the preparation isolated from the Hildenborough strain of *D. vulgaris* [6].

In this paper we describe a procedure for the purification of hydrogenase from the rumen organism *Megasphaera elsdenii*, a strictly anaerobic bacterium that produces hydrogen during growth on lactate or carbohydrates [16]. The molecular and catalytic properties of the pure enzyme have been examined and found to be closely similar to those of hydrogenase from *C. pasteurianum* [1]. A preliminary account of some of these results has been presented at a workshop on hydrogenases [17].

MATERIALS AND METHODS

Growth of Bacterium

Megasphaera elsdenii, strain LC 1 of Elsden et al. [16] (formerly named *Peptostreptococcus elsdenii* [18]), was grown on lactate/yeast extract according to the methods described by Walker [19] in iron-rich medium for the isolation of hydrogenase and ferredoxin, and in iron-poor medium for the isolation of flavodoxin [20].

Purification of Electron Carriers

Flavodoxin was purified from *M. elsdenii* by a method similar to that described by Mayhew and Massey [20]. The 8Fe ferredoxin in this organism [21, 22] was purified by a procedure similar to that described by Mortenson [23] for ferredoxin from *Clostridium pasteurianum*; the ratio of absorption, $A_{390\ nm}/A_{280\ nm}$, for the purified preparation was 0.79. Concentrations of flavodoxin and ferredoxin were determined from absorption measurements at 445 (ε_{445} = 10.2 $mM^{-1}\ cm^{-1}$) and 390 nm (ε_{390} = 30 $mM^{-1}\ cm^{-1}$) respectively.

Assays for Hydrogenase

Hydrogenase activity was routinely assayed by manometric measurement of hydrogen formation from dithionite-reduced methyl viologen [1, 24]. Warburg manometers contained in the main compartment, in a final volume of 2 ml: 150 µmol Tris-HCl buffer, pH 8, 2 µmol methyl viologen adjusted to pH 8, 100 µmol $Na_2S_2O_4$, 1 mg bovine serum albumin; the side arm contained 0.1–0.8 unit hydrogenase which, where necessary, had been diluted anaerobically in a mixture of 50 mM Tris-HCl buffer, pH 8, 2 mM $Na_2S_2O_4$ and bovine serum albumin (0.5 mg/ml); the centre well contained 0.2 ml 1 M NaOH; the gas phase was N_2 and the temperature was 30 °C. The sodium dithionite was dissolved anaerobically in 0.5 M Tris-HCl buffer pH 8 to give 1 M $Na_2S_2O_4$ and added to the main compartment of the manometer by syringe while flushing the manometer with N_2. The enzyme was added to the side arm under similar anaerobic conditions. The reaction was started by tipping the enzyme from the side arm into the main compartment.

Addition of bovine serum albumin (0.5 mg/ml) both to the assay and to the buffer used to dilute the enzyme is necessary in order to prevent inactivation of the hydrogenase with activity greater than about 50 units/mg. Ovalbumin has a similar stabilizing effect, indicating that the effect is not specific. The activity of cruder preparations of enzyme is not affected by adding protein to the assay.

The assay was used to study the effect of the concentration of methyl viologen on the rate of hydrogen production. The concentration of sodium dithionite was increased to 0.1 M when the concentration of methyl viologen exceeded 50 mM. It was assumed that this change in dithionite concentration does not change the rate of gas production because a similar increase in the dithionite in the standard assay with 1 mM methyl viologen has no effect on the initial rate.

Hydrogen oxidation activity of the enzyme was assayed by spectrophotometric measurement of the rate of reduction of methyl or benzyl viologen. Cuvettes that were stoppered with a Suba-seal rubber cap contained in a final volume of 1.5 ml: 100 µmol Tris-HCl buffer, pH 8, methyl viologen or benzyl viologen as indicated, 0.38 mg bovine serum albumin and an oxygen-scavenging system consisting of either 15 µmol glucose, and 0.37 mg glucose oxidase (Boehringer, grade 3; 7.4 units) or 1.5–7.5 µmol 2-mercaptoethanol plus a derivative of vitamin B_{12} that was obtained by treating vitamin B_{12} with HCl at 65 °C [25]. The cuvette and contents, but minus the glucose or B_{12} derivative, were made anaerobic by five cycles of evacuation and filling with hydrogen gas; after the final cycle, the cuvette was filled with hydrogen and then equilibrated at 30 °C. The pressure in the cuvette was adjusted to atmospheric pressure. The glucose or vitamin B_{12} derivative was then added by syringe, followed two minutes later by hydrogenase. The rate of reduction of methyl and benzyl viologens was determined at 604 nm and 540 nm respectively, using absorption coefficients of 13.6 $mM^{-1}\ cm^{-1}$ for the semiquinone of methylviologen [26] and 13.05 $mM^{-1}\ cm^{-1}$ for reduced benzyl viologen (S.G. Mayhew, unpublished results). Since both O_2-scavenging systems catalyse a slow reduction of viologen dyes, it was necessary to correct the observed rates for this blank activity.

Hydrogenase from *M. elsdenii* was prepared and stored in the presence of sodium dithionite; it was necessary to remove most of the strong reductant before adding the enzyme to the cuvette; this was achieved by first adding a trace of methyl viologen to the stock solution of enzyme and then adding aliquots of an anaerobic solution of potassium ferricyanide until the blue colour due to the reduced viologen was still just visible. Control experiments showed that this treatment does not cause inactivation of the enzyme as measured in the hydrogen-production assay.

As described in Results, the rate of reduction of benzyl viologen was also measured in the presence of high concentrations of salt (up to 1 M NaCl). It was observed that when reactions in the presence of high salt are allowed to proceed for periods much longer than required for initial rate measurements, a purple precipitate is formed in the solution. The nature of this precipitate was not investigated; it possibly results from polymerization of benzyl viologen semiquinone.

Determination of the Dissociation Constant of Methyl Viologen Semiquinone

Methyl viologen semiquinone is known to dimerize [27, 28]. Values for the dissociation constant of the dimer were determined at 30 °C in 75 mM Tris-HCl buffer pH 8 and 50 mM sodium dithionite as used in the hydrogen-production assay, and in similar mixtures but with the addition of salt. The determinations were made spectrophotometrically by measuring the absorbance at 875 nm as described by Thorneley [28], using absorption coefficients of 1.15 $mM^{-1}\,cm^{-1}$ and 6.44 $mM^{-1}\,cm^{-1}$ at 875 nm for the monomer and dimer respectively.

Chemical Analyses

Non-haem iron was determined by the method described by Massey [29]. Acid-labile sulphur was determined by the method described by Fogo and Popowsky [30] as modified by Brumby et al. [31]; the breakdown products of sodium dithionite in the enzyme solution interfere with the determination of acid-labile sulphur, and it was therefore necessary to use concentrated solutions of hydrogenase as recommended by Chen and Mortenson [32]. Protein was measured by the method of Lowry et al. [33] using bovine serum albumin as standard, and determining the albumin from its absorbance at 280 nm with an absorption coefficient of 436 $mM^{-1}\,cm^{-1}$ [34]. Protein was first precipitated with acetone/diethyl ether/trichloroacetic acid (16/4/1, v/v/w) to separate it from breakdown products of dithionite that interfere with the assay, and the precipitate was then washed with 5% (w/v) trichoroacetic acid before carrying out the analysis.

Determination of Molecular Weight

The molecular weight of hydrogenase was determined by gel filtration in a column of Sephadex G-100 [35] that had been calibrated with bovine serum albumin (M_r 67000), ovalbumin (M_r 44000), chymotrypsinogen (M_r 25700) and cytrochrome *c* (M_r 11700); an additional molecular weight determination was carried out by polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and sodium dodecyl sulphate [36] with glucose-6-phosphate dehydrogenase (M_r 100000), bovine serum albumin, ovalbumin and trypsin inhibitor from egg white (M_r 28000) as molecular weight markers.

Purification of Hydrogenase

The enzyme is inactivated by oxygen, and all operations during the purification procedure were therefore performed anaerobically by working in closed vessels with nitrogen as gas phase, and with 2–3 mM sodium dithionite present in the enzyme solution and all buffers. Column fractions were collected in glass bottles that were closed with a rubber septum and filled with N_2; solutions of enzyme were transferred by syringe. Dialysis was performed in closed vessels, flushed with nitrogen and containing 2–3 mM sodium dithionite. All operations were performed at 4 °C. An outline of the purification is given in Table 1.

Extraction of Bacteria. The cell paste of freshly harvested bacteria (1 kg) was suspended in 750 ml 0.1 M Tris-HCl buffer, pH 8, and after adding solid sodium dithionite to give a final concentration of 3 mM, the mixture was passed three times through a Manton-Gaulin homogeniser at 750 kg/cm^2 (73.5 MPa). Since centrifugation of the suspension of broken cells (20 min at 25000 × *g*) gave only a very small precipitate, the suspension was not centrifuged but was used directly for the following step.

Ammonium Sulphate Fractionation. A mixture of solid ammonium sulphate/solid Tris base (100/1.9, w/w), was added anaerobically to the broken cell suspension to give 60% saturation in ammonium sulphate, and stirred for 1 h. The Tris base was added to maintain the pH at 8. The suspension was centrifuged (30 min at 20000 × *g*), the precipitate was resuspended in 250 ml 0.1 M Tris-HCl buffer, pH 8, and the resulting turbid suspension was dialyzed for about 16 h versus 12 l 50 mM Tris-HCl buffer, pH 8, with a change of buffer after about 6 h. The dialyzed suspension was centrifuged (30 min at 20000 × *g*) and the black precipitate which was formed was discarded.

DEAE-cellulose Chromatography. The supernatant from the centrifugation step was loaded onto a column

(35 cm × 7 cm) of DEAE-cellulose (Whatman DE 32) that had been equilibrated with 50 mM Tris-HCl buffer, pH 8. The column was washed with 1 l 50 mM Tris-HCl buffer, pH 8, and the washings discarded. The column was then washed with 4 l 50 mM Tris-HCl buffer pH 8 plus 250 mM NaCl to elute the first of two bands of hydrogenase activity (fraction I). The salt in the buffer solution was then increased to 320 mM to elute the second band of activity (fraction II). The activity in fration II usually comprises about half of the activity applied to the column; this fraction was dialyzed overnight versus 10 l 15 mM sodium phosphate buffer, pH 8 with a change of buffer after 6 h. It should be noted that if the protein applied to the DEAE-cellulose column exceeds about 12 mg/ml of packed gel, the column becomes overloaded and a part of the hydrogenase activity corresponding to fraction I activity fails to bind. Further steps in the purification were carried out with fraction II.

First Treatment on Hydroxyapatite. The dialyzed enzyme was applied to a column (18 cm × 12 cm) of hydroxyapatite (Bio-Gel HTP from Bio-Rad) equilibrated with 15 mM sodium phosphate buffer, pH 8. The column was first washed with 250 ml 35 mM sodium phosphate buffer, pH 8, plus 35 mM NaCl, and then with a linear gradient of NaCl made by continuously diluting 500 ml 35 mM sodium phosphate buffer, pH 8, containing 35 mM NaCl with 500 ml 35 mM sodium phosphate buffer, pH 8, containing 500 mM NaCl. The eluted fractions contained much protein but no hydrogenase, and were discarded. The column was washed further with 750 ml 35 mM sodium phosphate buffer, pH 8, containing 500 mM sodium chloride followed by washing with 35 mM sodium phosphate buffer, pH 8, plus 35 mM NaCl (750 ml). The hydrogenase was removed from the column by a linear salt gradient made by continuously diluting 500 ml 35 mM sodium phosphate buffer, pH 8, containing 35 mM NaCl with 500 ml 125 mM sodium phosphate buffer, pH 8, containing 125 mM NaCl. The centre of the eluted peak of hydrogenase activity was at about 55 mM phosphate. The most active fractions were combined and concentrated to about 15 ml by ultrafiltration in an Amicon Diaflo apparatus equipped with a YM 10 membrane.

Gel Filtration. The concentrated enzyme fraction was applied to a column (125 cm × 3.5 cm) of Sephadex G-100 that had been equilibrated with 75 mM sodium phosphate buffer, pH 8, containing 750 mM NaCl. The column was developed with the same buffer, and fractions of about 15 ml were collected. This high concentration of NaCl in the buffer used for this column ensures that the enzyme is separated from material that has intense absorption at 257 nm; for instance at 100 mM NaCl, the 257-nm-absorbing material is still associated with the hydrogenase-containing fractions. The most active fractions from the gel filtration step were combined and dialyzed overnight versus 8 l 10 mM sodium phosphate buffer pH 7.6.

Second Treatment on Hydroxyapatite. The dialyzed solution was added to a second column of hydroxyapatite (10 cm × 3.5 cm) equilibrated with 35 mM sodium phosphate buffer, pH 7.6. The column was washed with 200 ml of the phosphate buffer before eluting it with a linear gradient made by diluting 500 ml 35 mM sodium phosphate buffer, pH 7.6, with 500 ml 250 mM sodium phosphate buffer, pH 7.6. The centre of the peak of activity from the column was at about 60 mM phosphate. The most active fractions from the column were combined, and the enzyme stored in liquid nitrogen.

Purification of Gases

Nitrogen and hydrogen gases were freed of oxygen by passing them through a column of BASF catalyst at about 120 °C, followed by passage through a wash bottle that contained a solution of methyl viologen semiquinone.

Analysis of Data

Computer fits were obtained by adjusting the parameters of the theoretical functions with an iterative weighted least-square-fit procedure to the experimental kinetic data.

RESULTS AND DISCUSSION

EXTRACTION AND PURIFICATION OF HYDROGENASE

The hydrogenase activity of *Megasphaera elsdenii* was extracted from the bacterium by breaking the cells in a Manton-Gaulin homogenisor. Attemps were made to extract the activity from whole cells by a variety of milder treatments, including incubation with Tris plus EDTA, that have been used to extract periplasmic proteins from other gram-negative bacteria. Activity was not extracted by these methods, a result which suggests that in contrast to the enzyme in *Desulfovibrio vulgaris* strain Hildenborough [6], and in *Desulfovibrio gigas* [7,37], the hydrogenase of *M. elsdenii* is not located in the cell envelope. Cells of *M. elsdenii* are surrounded by a thick coat of carbohydrate, however [38], and it is possible therefore, that even if hydrogenase is located in the cell envelope, the thick outer layer may prevent its removal by the non-disruptive methods that were tested. The hydrogenase activity in a cell-free extract of cells broken as described does not precipitate when the extract is centrifuged at 200000 × *g* for 90 min, indicating that the enzyme(s) (see below) are in a soluble form, as also was found by Joyner et al. [39].

As summarized in Table 1, the purification leads to a 200-fold increase in the specific activity with a

Table 1. *Purification of a hydrogenase from* Megasphaera elsdenii
The crude extract was prepared from 1 kg bacteria (wet weight) grown in an iron-rich medium [20]. Activity was measured in the standard hydrogen-production assay, as described in Materials and Methods

Step	Volume	Total protein	Total activity	Specific activity	Recovery of activity
	ml	mg	units	units/mg	%
Crude extract	2500	92000	228000	2.48	100
Ammonium sulphate fractionation 0–60% saturation	2330	59000	187000	3.17	82
DEAE-cellulose					
Fraction I (0.2 M NaCl)	2950	41900	35000	0.84	15
Fraction II (0.32 M NaCl)	2830	1700	89600	52.7	39
Fraction II:					
First hydroxyapatite	652	273	66300	243	29
Sephadex G-100	185	116	48700	419	21
Second hydroxyapatite	202	64.3	32800	510	14

yield of activity of 14%. The enzyme as eluted from the second column of hydroxyapatite gives a single protein band upon electrophoresis on polyacrylamide gel [36]; a single band is also observed when the electrophoresis is carried out in the presence of sodium dodecyl sulphate and 2-mercaptoethanol to denature the protein. The concentration range of purified hydrogenase used for electrophoresis on polyacrylamide gel in the absence and presence of sodium dodecyl sulphate and 2-mercaptoethanol was 10–75 μg We conclude, therefore, that the final preparation is homogeneous.

A large part of the total activity in the original extract is separated in fraction I from the DEAE-cellulose column. The activity in this fraction differs from that in fraction II in its behaviour on DEAE-cellulose, hydroxyapatite, during gel filtration and also in its kinetic properties of the hydrogen production with methyl viologen and flavodoxin as electron donors and the hydrogen uptake with methyl viologen as electron acceptor. Its elution volume from a calibrated column of Sepharose 4B indicated that it corresponds with a protein of $M_r \approx 200000$ and that it is therefore a larger molecule than the enzyme purified from fraction II (see below). Attempts to obtain the enzyme in fraction I in a highly purified form have not yet been successful however, and it is therefore not clear whether it represents a different hydrogenase molecule or simply a complex of the enzyme in fraction II.

The pure enzyme from fraction II is stored in liquid nitrogen, since it is stable indefinitely under these conditions.

PHYSICO-CHEMICAL PROPERTIES

Values for the molecular weight of the enzyme were obtained from measurements of its elution volume from a calibrated column of Sephadex G-100 [36] and from polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulphate and 2-mercaptoethanol [36]. The values determined by the two methods are 49000 and 52000 respectively; the close agreement of the value obtained under the denaturing conditions of the gel electrophoresis with the value from gel filtration indicates that the enzyme consists of a single polypeptide chain.

Chemical analyses for non-haem iron and acid-labile sulphur gave 11.7 ± 1.3 mol Fe · mol^{-1} and 11.5 ± 0.9 mol S^{2-} · mol^{-1}. The iron and sulphide content of this hydrogenase is therefore similar to those of the hydrogenases from *Clostridium pasteurianum* [1,15], *D. vulgaris* strain Hildenborough [6] and *D. gigas* [7], all of which contain about 12 mol Fe · mol^{-1} and 12 mol S^{2-} · mol^{-1}.

The enzyme as it is isolated is yellow-brown and it shows a featureless tail of absorption throught the visible spectrum, as is typical of proteins which contain reduced Fe_4-S_4 redox chromophore(s) [40]. The protein absorbance in the ultraviolet region is obscured by the absorbance of sodium dithionite. Treatment of the preparation with air causes oxidation of the dithionite and the visible absorbance of the protein subsequently increases. The spectrum of this oxidized form of the enzyme shows a maximum at 275 nm a broad shoulder between 350 nm and 400 nm. The ratio $A_{400\,nm}/A_{275\,nm}$ is 0.32. The absorption coefficient at 400 nm for the air-oxidized enzyme is 46 mM^{-1} cm^{-1}, and it is therefore similar to published values for 12Fe-12S-containing hydrogenases from other anaerobic bacteria [1,6,7]; the absorbance of the enzyme as it is isolated is 14% lower. The visible absorbance of the air-treated enzyme decreases by 75% within 2 h at room temperature.

CATALYTIC PROPERTIES

Hydrogen Production with Ferredoxin and Flavodoxin

Previous work with crude preparations of hydrogenase suggested that the direct electron donor for

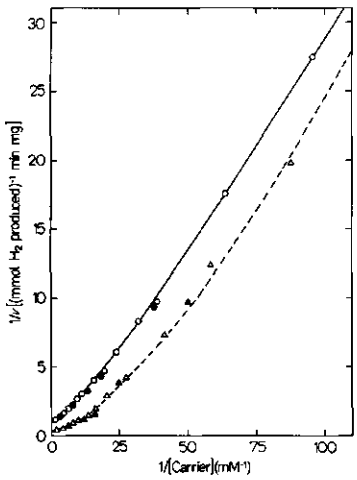

Fig. 1. *Double-reciprocal plots of the hydrogen production activity, v, on the concentration of fully reduced* M. elsdenii *flavodoxin and ferredoxin.* With ferredoxin as electron donor the total volume in the main compartment of the Warburg manometer was 1 ml. (○) Observed initial activity with flavodoxin; (●) observed initial activity with flavodoxin plus 1 mM methyl viologen, after correction for the partial contribution of the production activity with methyl viologen; (——) calculated activity with flavodoxin according to Eqns (6) or (7); (△) observed initial activity with ferredocin; (▲) observed initial activity with ferredoxin plus 1 mM methyl viologen, after correction for the partial contribution of the production activity with methyl viologen; (– – –) calculated activity with ferredoxin according to Eqns (6) or (7)

the enzyme in *M. elsdenii* is either an 8Fe-8S ferredoxin in bacteria grown in iron-rich medium [21] or the FMN protein flavodoxin in cells from iron-poor medium [41]. This conclusion is supported by experiments which show that the highly purified enzyme from iron-rich cells catalyzes hydrogen production from dithionite with either of the electron carriers. As has been reported elsewhere [17] the enzyme shows no activity with cytochrome c_3, a low-potential electron carrier that is believed to be the electron donor for hydrogenase in *D. vulgaris*. Neither ferredoxin nor flavodoxin sature the *M. elsdenii* enzyme at the highest concentrations tested, and double-reciprocal plots of the substrate saturation curves are not linear (Fig. 1). Hill plots for flavodoxin and ferredoxin, using values for V of 1000 units · mg^{-1} and 4160 units · mg^{-1} respectively show values for h of 1.3 and 1.4; the apparent $[S]_{0.5}$ values are 0.15 mM and 0.28 mM respectively. Chen and Mortenson [1, 14] observed that when a mixture of ferredoxin and methyl viologen was used with *C. pasteurianum* hydrogenase, the rate of hydrogen production was larger than the sum of the rates observed when the electron carriers were tested separately. We have not observed a similar effect with *M. elsdenii* hydrogenase; the rates with ferredoxin plus methyl viologen (1 mM) and flavodoxin plus methyl viologen (1 mM) are additive when the concentrations of ferredoxin and flavodoxin are in the range of 0.2–3 times the apparent $[S]_{0.5}$ values.

Sodium chloride stimulates hydrogen production from dithionite with methyl viologen as electron carrier (see below). However, it inhibits the reaction with ferredoxin and flavodoxin. At concentrations of the electron carriers equal to the apparent $[S]_{0.5}$ values the rates of hydrogen production with ferredoxin and flavodoxin are halved at an NaCl concentration of 0.5 M and 0.2 M respectively. It is possible that a complex of hydrogenase and carrier is disrupted by a high salt concentration, as has been observed for the protein-protein complex of ferredoxin-NADP$^+$ reductase and ferredoxin [42].

Hydrogen Production with Methyl Viologen

The rate of hydrogen production from dithionite with methyl viologen as electron carrier varies with the methyl viologen concentration, the pH, and is also affected by a variety of salts. It was noticed that during the period of measurement of hydrogen production in the standard assay the activity of the enzyme increased after about 3 µmol hydrogen had been produced. Dithionite is oxidised during the reaction, resulting in the formation of stoichiometric amounts of hydrogen and a fall in the pH of the reaction mixture. The buffer concentration in the assay was not sufficient to maintain the pH at 8; below this pH, the extent of reduction of methyl viologen by dithionite decreases [43]. This acidification process can be described by the following set of equations:

$$^1/_2\, S_2O_4^{2-} \overset{K_a}{\rightleftharpoons} SO_2^- \qquad (1)$$

$$SO_2^- + H_2O \rightleftharpoons HSO_3^- + H^+ + e^- \qquad (2)$$

$$HSO_3^- \overset{pK}{\rightleftharpoons} SO_3^{2-} + H^+ \qquad (3)$$

$$H^+ + e^- \overset{\text{hydrogenase}}{\rightleftharpoons} {}^1/_2\, H_2\,. \qquad (4)$$

The overall reaction is given by:

$$^1/_2\, S_2O_4^{2-} + H_2O \overset{\text{hydrogenase}}{\rightleftharpoons} SO_3^{2-} + H^+ + {}^1/_2\, H_2\,. \qquad (5)$$

The value for the association constant of reaction (1) is: $K_a = 7.14 \times 10^8$ M^{-1} [44], the pK value for the ionization of bisulphite is 6.9 [45].

The relationship between the amount of hydrogen produced, the pH, the concentration of methyl viologen semiquinone and the enzyme activity is shown in Fig. 2 A.

The observed change in activity is probably the result of two effects: an increase in the rate of hydrogen production due to a decrease in pH, and a decrease in rate due to a decrease in the concentration of

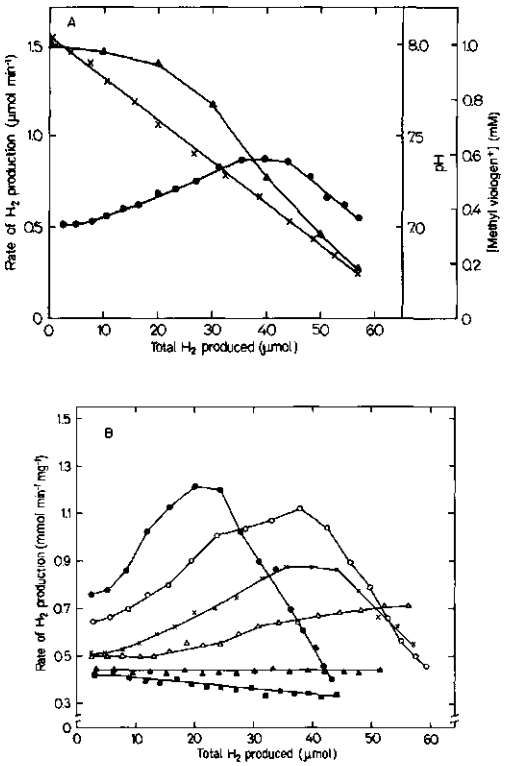

Fig. 2. *Dependence of (A) the hydrogen-production activity, the pH and the concentration of methyl viologen semiquinone in the standard assay and (B) the hydrogenase evolution activity on the total amount of H_2 produced.* The hydrogenase activity was measured as described in Materials and Methods, in the presence of 1 mM methyl viologen as electron carrier. (A) The pH was measured in a glass bottle which had the same volume as the Warburg vessel and contained the same reaction mixture. The bottle was sealed with a rubber septum through which a pH electrode was brought into the reaction mixture. The system was flushed with N_2 and the reaction was started by injecting the hydrogenase preparation into the reaction mixture. The bottle plus electrode was shaken identically to the Warburg manometer. It was checked, after the assay was finished, that the pH of the Warburg vessel was the same as in the glass bottle. The concentration of reduced methyl viologen was calculated according to Mayhew [43]. The amount of hydrogenase added to the Warburg vessel and glass bottle was 1.18 μg. (●——●) rate of H_2 production; (×——×) pH; (▲——▲) methyl viologen semiquinone concentration. (B) The concentration of the Tris-HCl buffer pH 8 was varied: (●——●) 30 mM; (○——○) 50 mM; (×——×) 75 mM; (△——△) 150 mM; (▲——▲) 350 mM; (■——■) 500 mM

methyl viologen semiquinone. The change in rate was also measured at several concentrations of Tris-HCl buffer, pH 8 (Fig. 2B). It appears that the initial activity decreases with increasing buffer concentrations, but the acceleration of hydrogen production disappears. An increase of rate due to the decrease of

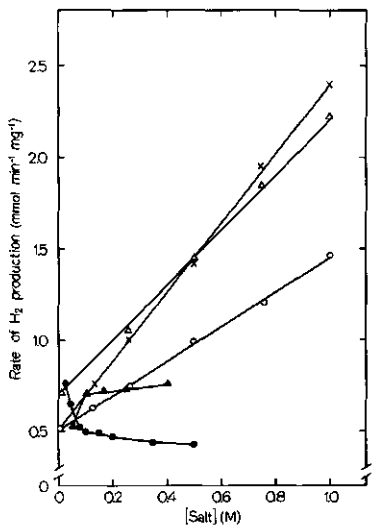

Fig. 3. *Dependence of the hydrogen-production activity on the concentration of buffer, NaCl and $MgCl_2$.* Hydrogen-production activity was measured as described in Materials and Methods with 1 mM methyl viologen semiquinone as electron carrier. (●——●) Initial hydrogen production activity with varying concentrations Tris-HCl buffer, pH 8; (○——○) initial evolution activity in the standard production assay, with increasing amounts of sodium chloride; (×——×) initial evolution activity in the standard production assay with increasing amounts of $MgCl_2$; (▲——▲) initial production activity in the presence of various concentrations of sodium phosphate buffer, pH 8.0; (△——△) initial activity in sodium phosphate buffer, pH 8.0 (140 mM) at increasing concentrations of sodium chloride

pH was also observed with ferredoxin and flavodoxin as electron carriers.

Salts such as sodium phosphate, pH 8, NaCl and $MgCl_2$ cause an increase in activity; the increase is linear with the concentration of NaCl and $MgCl_2$ (Fig. 3). The apparent specific activity of the enzyme after addition of 1 M NaCl to the assay with 1 mM methyl viologen is 1470 units · mg^{-1} compared with a value of 510 units · mg^{-1} in the absence of the salt. The stimulation observed with 1 M $MgCl_2$ (1890 units · mg^{-1}) is twice that by 1 M NaCl, suggesting that the effect is due to the anion. The inhibition by Tris-HCl buffer has previously been observed with preparations of hydrogenases from other sources [4, 14].

The effect of the concentration of methyl viologen on the rate of hydrogen production was examined in detail by using the standard assay mixture with and without the addition of NaCl. The highest concentration of methyl viologen tested (70 mM) does not saturate the enzyme, and double-reciprocal plots of the substrate saturation curves are not linear (Fig. 4). Hill plots of the data in the absence and presence of NaCl (1.0 and 1.5 M) show two linear regions of

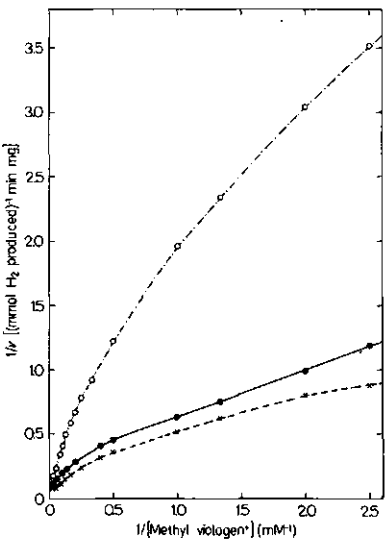

Fig. 4. *Double-reciprocal plots of the hydrogen-production activity, v, of* M. elsdenii *hydrogenase on the concentration of reduced methyl viologen in the absence and presence of sodium chloride.* (○) Observed activity in absence of NaCl, (·–·–·) activity calculated according to Eqn (7); (●) observed activity at 1 M NaCl, (——) activity calculated according to Eqn (7); (×) observed activity at 1.5 M NaCl, (– – –) activity calculated according to Eqn (7)

different slope. In all three cases below 5 mM methyl viologen h is about 0.7; above a dye concentration of 8 mM h is about 1.5.

Sodium dithionite is always present in excess in the assay, and the methyl viologen is therefore present in its semiquinone form. This reduced form is known to dimerize with a dissociation constant of about 2 mM [27,28]. It is possible that the emzyme has different kinetic constants for the monomer and dimer, and that the monomer-dimer equilibrium accounts at least in part for the non-linearity in the double-reciprocal plots and for the biphasic Hill plots. At concentrations of methyl viologen below 5 mM the monomer is probably the main substrate and at concentrations above 8 mM monomer and dimer probably both serve as substrate.

It was observed that the colour of methyl viologen semiquinone changed from blue to purlpe as the NaCl concentration was increased. Quantitative determination of the dissociation constant as described in Methods showed that high salt favours formation of the dimer. In 0, 0.1, 1.0 and 1.5 M NaCl the values determined for K_d are 8.6, 5.3, 1.2 and 0.85 mM respectively.

As has been reported elsewhere [17] *D. vulgaris* hydrogenase shows saturation kinetics up to about 2 mM methyl viologen, but the rate further increases with increasing methyl viologen to give a maximum activity at about 15 mM of the dye. This effect supports the idea that the hydrogenases of *M. elsdenii* and *D. vulgaris* have different kinetic constants for the monomer and dimer forms of methyl viologen semiquinone.

Hydrogen Production without an Electron Carrier

Purified hydrogenase produces hydrogen from dithionite even in the absence of an electron carrier, but the activity is low (2.0 μmol H_2 min^{-1} mg^{-1}). The activity increases by addition of NaCl to give values of 5.1, 8.2 and 11.2 μmole H_2 min^{-1} mg^{-1} in 0.5 M, 1.0 M and 1.5 M NaCl respectively. It is known that solutions of sodium dithionite contain small amounts of SO_2^- in eqilibrium with dithionite ion ($S_2O_4^{2-}$) [44]. High salt concentrations may increase the dissociation constant of this equilibrium, and if SO_2^- can serve as substrate the increase in its concentration might account for the observed increase in activity. However, an effect of salt on the hydrogenase itself, resulting in an increased activity in the presence of salt, cannot be excluded because the hydrogen oxidation activity is also stimulated by addition of NaCl (see below).

Hydrogen Oxidation with Methyl Viologen and Benzyl Viologen as Electron Acceptors

The enzyme has been shown to catalyse the oxidation of molecular hydrogen with ferredoxin, flavodoxin, methyl viologen and benzyl viologen, FMN, 2,6-dichloroindophenol and methylene blue as electron acceptors, but not with $NAD(P)^+$ (2 mM). The spectrophotometric assay described in Materials and Methods was used to study the effect of varying concentrations of the viologen dyes on the hydrogenase activity. A double-reciprocal plot of the kinetic data for benzyl viologen was linear in the range 0.1 – 45 mM of the dye concentration; the V is 9000 μmol · min^{-1} · mg^{-1} and the K_m value is 5.7 mM. The Lineweaver-Burke plot for the kinetic data for methyl viologen is also linear for the concentration range of 0.1 – 80 mM. The V is 42000 μmol · min^{-1} · mg^{-1} with a very high K_m value of 171 mM.

In contrast to its inhibitory effect on the hydrogen production reaction, Tris-HCl buffer, pH 8, was found to stimulate hydrogen oxidation with methyl or benzyl viologen as electron acceptor (Fig. 5 A and B). Sodium chloride and magnesium chloride also enhance the activity with methyl or benzyl viologen, and the increase is linear with the salt concentrations up to about 0.5 M. The rate continues to increase at higher salt concentrations, but the increase is no longer linear. It is possible that the non-linearity at high salt concentrations is due to a decreasing solubility of hydrogen gas [46].

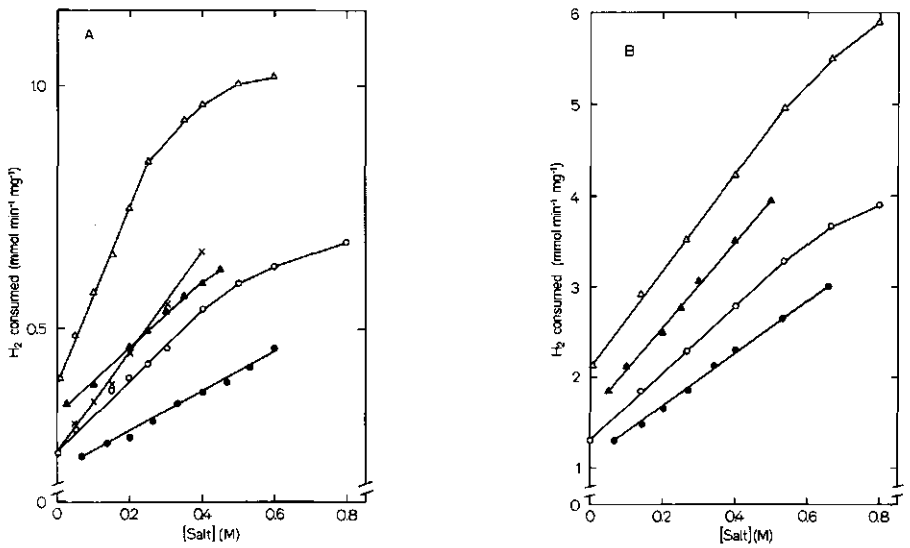

Fig. 5. *Dependence of the hydrogen-oxidation activity on the concentration of buffer, NaCl and $MgCl_2$.* (A) Hydrogen-oxidation activity was measured as described in Materials and Methods with 1 mM methyl viologen as electron acceptor. (●——●) Initial hydrogen-oxidation activity with varying concentrations Tris-HCl buffer, pH 8; (○——○) initial consumption activity in the standard assay with increasing amounts of NaCl; (×——×) initial activity in the standard assay with increasing amounts of $MgCl_2$; (▲——▲) initial consumption activity in the presence of various concentrations of sodium phosphate buffer, pH 8.0; (△——△) initial activity in sodium phosphate buffer, pH 8.0 (100 mM) at increasing concentrations of NaCl. (B) Hydrogen oxidation activity with 1 mM benzyl viologen as electron acceptor. Symbols are as for (A)

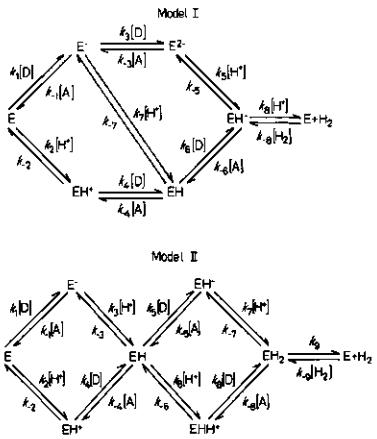

Fig. 6. *Models for* M. elsdenii *hydrogenase activity.* k_1, k_{-1}, k_2, etc. are rate constants; [D] reduced electron-donor concentration; [A] oxidized electron-acceptor concentration; E, hydrogenase enzyme; E^-, one-electron-reduced enzyme; EH^+, enzyme-proton complex; EH, enzyme–native-hydrogen complex; E^{2-}, fully reduced enzyme; EH^-, enzyme-hydride complex

COMPUTER FITTING OF KINETIC DATA

As has been pointed out by Atkinson and Walton [47] and confirmed by the kinetic models of Ferdinand [48], Fisher and Hoogland [49] and Sweeny and Fisher [50], kinetic patterns showing apparent co-operativity can be obtained with two-substrate enzymes which have one catalytic centre. Because the hydrogenase consists of a single polypeptide chain, and no indication was obtained of a higher order of enzyme complex, the kinetic data for hydrogen production were further analyzed to obtain a kinetic model which does not involve co-operativity. Two kinetic models were developed which are consistent with the data (Fig. 6). It could be shown that models in which the enzyme reacts in a compulsory way with a reduced electron donor and a proton, give rate equations which cannot fit the data. The general rate equation for the two models for the initial hydrogen production is:

$$\frac{v}{[E]_t} = \frac{x_1[D] + x_2[D]^2 + x_3[D]^3}{x_4 + x_5[D] + x_6[D]^2 + [D]^3} \qquad (6)$$

in which $[E]_t$ is the total enzyme concentration, [D] is the concentration of the reduced electron donor and x_i is a set of rate constants for the individual steps, taking into account that the proton concentration is kept constant at 10 nM.

The results of fitting the kinetic data for reduced flavodoxin and ferredoxin according to Eqn (6) are given in Table 2. As can be seen in this table, the contributions of $x_1[D]$ and x_4 are relatively small especially at donor concentrations exceeding 0.1 mM. This

Table 2. *Results of fitting the kinetic data for flavodoxin and ferredoxin according to Eqn (6)*
$x_1 - x_6$ are rate constants according to Eqn (6). The concentration range given is that of the electron donor used to test the hydrogenase activity; n is the number of experiments performed at different donor concentrations

Parameter	Unit	Value for	
		flavodoxin	ferredoxin
x_1	$mM^3\ min^{-1}$	0.0151	0
x_2	$mM^2\ min^{-1}$	0.870	4.16
x_3	$mM\ min^{-1}$	0.995	1.73
x_4	mM^3	0.0053	0.074
x_5	mM^2	0.150	0.345
x_6	mM	0.903	0.600
$[S]_{0.5}$	mM	0.158	0.277
V	$mmol\ min^{-1}\ mg^{-1}$	0.995	4.16
Standard error estimate	%	0.6	0.8
Concentration range	mM	0.003–0.63	0.005–0.58
Substrate inhibition		–	–
n		17	17

is the case if in both models one step is relatively slow. For model I (Fig. 6) this occurs if one of the rate constants $k_1 - k_6$ or both rate constants k_7 and k_{-7}, or in model II (Fig. 6) one of the rate constants $k_1 - k_8$ is very small. Therefore, neglecting $x_1[D]$ and x_4, Eqn (6) simplifies to the following rate equation:

$$\frac{v}{[E]_t} = \frac{x_2[D] + x_3[D]^2}{x_5 + x_6[D] + [D]^2} . \qquad (7)$$

Computer simulations of the data for flavodoxin and ferredoxin according to Eqn (7) fit almost as well as those obtained with Eqn (6) (cf. standard error of estimate in Table 3).

For both models it can be concluded, firstly, that oxidized (E) or reduced (E^-) hydrogenase reacts with a proton; if this were not so Michaelis-Menten kinetics would be observed. Presumably the proton is in the immediate vicinity of its electron-accepting site(s). Secondly, because of the low proton concentration the rate constants connected with the proton are at least ten times greater than those connected with the carrier.

For both models it can be shown theoretically that a mixture of two carriers will give the sum of the activities of the individual carriers. This is confirmed by the results with flavodoxin plus methyl viologen and ferredoxin plus methyl viologen. Methyl viologen semiquinone consists of a mixture of the semiquinone monomer and the dimer form [27, 28]. It is assumed that the activities with the monomer and dimer forms are also additive and that both activities fulfill Eqns (6) or (7) but with different values for the kinetic constants x_i.

Eqn (7) was used to describe the activity towards methyl viologen, in the absence and presence of NaCl; it is less complicated than Eqn (6) and it fits the data for flavodoxin and ferredoxin almost as well (Table 3). As can be seen from Fig. 4 the theoretical curves show a good fit with the experimental data.

The results of fitting the activities with methyl viologen, flavodoxin and ferredoxin according to Eqn (7) are collected in Table 3. Several conclusions can be drawn from Tables 2 and 3. The natural two-electron donor ferredoxin shows identical kinetic behaviour compared with the one-electron donors flavodoxin (natural) and methyl viologen semiquinone monomer (artificial). This means that the hydrogenase accepts two electrons in two separate steps from flavodoxin and methyl viologen. Because of the identical kinetic behaviour towards ferredoxin the hydrogenase also accepts the electrons in two discrete one-electron transfer steps from this electron donor. This indicates that the two (4Fe-4S) clusters within the ferredoxin are independent.

The conclusion that the ferredoxin releases one electron per step can probably also be extended to the artificial donor methyl viologen semiquinone dimer. The calculated partial contributions of the monomer and dimer to the hydrogenase activity are shown in Fig. 7 A and B respectively. It is shown, that from 0 up to 1.0 M NaCl the increase in activity can mainly be ascribed to the monomer, while from 1.0 M up to 1.5 M NaCl the further increase in activity is due to the dimer.

The dimer shows substrate inhibition (cf. Table 3 and Fig. 7 B); this might be explained by the fact that methyl viologen semiquinone not only dimerizes, but possibly forms still larger complexes [51]. Such polymerisation products might also react with the enzyme but with different values for the kinetic constants. On the other hand substrate inhibition can also be observed if a preferential pathway becomes less important because of an increase in concentration of a substrate, as has been shown by Ferdinand [48].

Electron paramagnetic resonance (EPR) experiments (to be published) show that the hydrogenase contains three (4Fe-4S) clusters of which two are of the ferredoxin type and one of the high-potential (Hipip) type. The EPR spectra of the reduced enzyme and the oxidized enzyme are very similar to the corresponding EPR spectra of *C. pasteurianum* hydrogenase [2, 13, 14]. The EPR spectra of our reduced enzyme, reduced with dithionite or H_2 (E^{2-} and EH^- in model I; EH^- and EH_2 in model II), do not show proton splitting, indicating that the reduced enzyme is mainly in the E^{2-} form. This favours model I compared to model II and further indicates that in the first model the ratio $k_5[H^+]/k_{-5}$ is very small. It can be shown that at low electron-donor concentrations the formation of an enzyme-proton complex is domi-

Table 3. *Results of fitting the kinetic data according to Eqn (7) for flavodoxin, ferredoxin and for methyl viologen in the absence and presence of sodium chloride*
x_i are rate constants according to Eqn (7). Other details as in Table 2

Parameter	Unit	Value for							
		flavodoxin (no NaCl)	ferredoxin (no NaCl)	methyl viologen semiquinone					
				monomer at NaCl			dimer at NaCl		
				0	1.0 M	1.5 M	0	1.0 M	1.5 M
x_2	$mM^2\ min^{-1}$	0.0244	0.0405	0.425	10.6	20.3	4.38	48.4	13.1
x_3	$mM\ min^{-1}$	1.066	4.16	3.62	7.25	7.16	3.36	2.74	4.18
x_5	mM^2	0.0083	0.0177	0.0627	2.67	3.74	37.5	167	110
x_6	mM	0.171	0.234	5.80	4.15	6.68	0.0062	0	3.60
$[S]_{0.5}$	mM	0.148	0.281	5.44	2.11	2.54	5.75	3.32	2.97
V	$mmol\ min^{-1}\ mg^{-1}$	1.07	4.16	3.62	7.25	7.16	3.41	3.69	7.44
Standard error estimate	%	0.8	1.3	0.8[a]	2.2[a]	1.4[a]	0.8[a]	2.2[a]	1.4[a]
Concentration range	mM	0.003–0.63	0.005–0.58	0.1–70[b]	0.1–45[b]	0.1–40[b]	0.1–70[b]	0.1–45[b]	0.1–40[b]
Substrate inhibition		–	–	–	–	–	+	+	+
n		17	17	24	19	19	24	19	19

[a] Calculated for the sum of the monomer and dimer activities at 0, 1.0 and 1.5 M NaCl respectively.
[b] This range is given for the concentration of this dye without taking the dimerization into account.

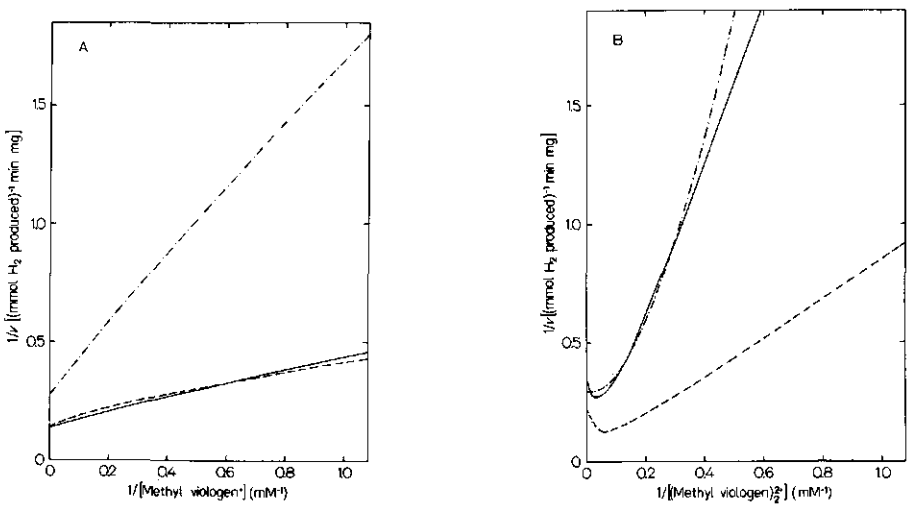

Fig. 7. *Computer fittings according to Eqn (7) on the concentration of methyl viologen semiquinone monomer (A) and dimer (B) in the absence and presence of NaCl.* (·—·) Fitting in absence of NaCl; (——) fitting at 1 M NaCl; (– – –) fitting at 1.5 M NaCl

nant for both models. This favours model II because model I shows a deprotonating step ($EH^- \rightarrow E^-$) which is assumed to be fast. Therefore no choice can be made between the two models at present.

We propose that the ferredoxin-type clusters each randomly react with an electron-donor and a proton. However, the mechanism of the formation of hydrogen from this fully reduced enzyme-proton complex and the function of the Hipip-type cluster is not yet clear. Recently Chen [52] proposed two mechanisms for the hydrogenase of *C. pasteurianum*; in our opinion neither of these mechanisms can explain the published kinetic data [1, 53]. The proposed mechanism I of Chen [52] predicts that the rate will depend on the square of the concentration of the electron donor, but the double-reciprocal plots were linear [1, 53]. His mechanism II closely resembles our model II; Michaelis-Menten kinetics for this model can only be obtained

if the enzyme reacts compulsory with the electron donors and protons. It should be pointed out, however, that the concentration range of ferredoxin tested with *C. pasteurianum* hydrogenase was quite narrow and might not have been wide enough to observe the non-linearity we find with our enzyme.

As mentioned earlier the kinetics of hydrogen oxidation show Michaelis-Menten kinetics. This kinetic behaviour can be explained by the two models (Fig. 6) if it is assumed that the reaction mechanism for the hydrogen oxidation is compulsory. This means that in model I the pathway will be $E \rightarrow EH^- \rightarrow E^{2-} \rightarrow E^- \rightarrow E$ assuming that $k_7[H^+]/k_{-7}$ is very small. In modell II the exact order of intermediates cannot be specified.

COMPARISON WITH HYDROGENASES FROM OTHER ANAEROBES

The purified hydrogenase from *M. elsdenii* can be compared with enzyme preparations obtained from the strictly anaerobic bacteria *C. pasteurianum* [1, 2], *D. vulgaris* strain Hildenborough [6] and *D. gigas* [7]. At least two different preparations of hydrogenase have been reported for each of these three bacteria, the physico-chemical properties of the purified enzymes varying with the preparation. All of the most recent preparations reported, however, contain about 12 atoms of iron and an equivalent amount of acid-labile sulphide, similar to that found in the preparation from *M. elsdenii*, and, with the exception of the preparation from *D. gigas* which contains an additional subunit of molecular weight 26000, the enzymes consist of a single subunit in the molecular weight range 50000 – 60000.

The catalytic properties of the enzyme from *M. elsdenii* seem to resemble closely those of hydrogenase from *C. pasteurianum*. Both enzymes show high activity in the hydrogen-production assay with methyl viologen and either ferredoxin or flavodoxin; in contrast to the enzymes from *D. vulgaris* and *D. gigas*, they fail to catalyze hydrogen production from dithionite-reduced cytochrome c_3. These two enzymes further resemble one another in being rapidly inactivated in air. The kinetics of reaction of *C. pasteurianum* hydrogenase with methyl viologen has recently been examined by Erbes and Burris [53] who also used manometric and spectrophotometric assays to measure hydrogen production and oxidation respectively. They observed linear double-reciprocal plots for the hydrogen production assay when the methyl viologen concentration was varied. However, the range of concentration which they examined was not wide (0.08 mM to 2.5 mM methyl viologen) and this range may not have been sufficient to observe irregularities similar to those reported in this paper for the enzyme from *M. elsdenii*.

APPENDIX

x_i *as a Function of* k_i, *the Proton Concentration and the Hydrogen Concentration*

In both models for the hydrogen production the electron-donor concentration [D] is variable, the proton concentration is constant and the electron-acceptor concentration [A] and hydrogen concentration are zero.

In both models for the hydrogen oxidation the electron-acceptor concentration is variable, the hydrogen and proton concentrations are constant and the electron-donor concentration is zero.

Model I: Hydrogen Production

$$\text{Denominator} = k_1k_3k_4k_{-5}k_6 + (k_1k_3k_4k_5k_6 + k_1k_3k_4k_6k_8)[H^+].$$

$$x_1 = \{(k_1k_{-2}k_3k_5k_{-7}k_8)[H^+]^2 + (k_1k_{-2}k_5k_6k_7k_8 + k_2k_3k_4k_5k_{-7}k_8)[H^+]^3 + (k_2k_4k_5k_6k_7k_8)[H^+]^4\}/\text{denominator}.$$

$$x_2 = \{(k_1k_{-2}k_3k_5k_6k_8 + k_1k_3k_4k_5k_{-7}k_8)[H^+]^2 + (k_1k_4k_5k_6k_7k_8 + k_2k_3k_4k_5k_6k_8)[H^+]^3\}/\text{denominator}.$$

$$x_3 = (k_1k_3k_4k_5k_6k_8)[H^+]/\text{denominator}.$$

$$x_4 = \{(k_1k_{-2}k_5k_{-7}k_8 + k_{-2}k_3k_5k_{-7}k_8 + k_3k_4k_5k_{-7}k_8)[H^+]^2 + (k_1k_{-2}k_5k_7k_8 + k_2k_3k_5k_{-7}k_8 + k_2k_4k_5k_{-7}k_8 + k_{-2}k_5k_6k_7k_8)[H^+]^3 + (k_2k_4k_5k_7k_8 + k_2k_5k_6k_7k_8)[H^+]^4\}/\text{denominator}.$$

$$x_5 = \{k_1k_{-2}k_3k_{-5}k_{-7} + (k_1k_{-2}k_3k_5k_{-7} + k_1k_{-2}k_3k_{-7}k_8 + k_1k_{-2}k_{-5}k_6k_7 + k_2k_3k_4k_{-5}k_{-7})[H^+] + (k_1k_{-2}k_5k_6k_7 + k_1k_{-2}k_5k_6k_8 + k_1k_4k_5k_{-7}k_8 + k_2k_3k_4k_5k_{-7} + k_2k_3k_4k_{-7}k_8 + k_2k_4k_{-5}k_6k_7 + k_{-2}k_3k_5k_6k_8)[H^+]^2 + (k_1k_4k_5k_7k_8 + k_2k_3k_4k_5k_8 + k_2k_3k_5k_6k_8 + k_2k_4k_5k_6k_7 + k_4k_5k_6k_7k_8)[H^+]^3\}/\text{denominator}.$$

$$x_6 = \{k_1k_{-2}k_3k_{-5}k_6 + k_1k_3k_4k_{-5}k_{-7} + (k_1k_{-2}k_3k_5k_6 + k_1k_{-2}k_3k_6k_8 + k_1k_3k_4k_5k_{-7} + k_1k_3k_4k_{-7}k_8 + k_1k_4k_{-5}k_6k_7 + k_2k_3k_4k_{-5}k_6)[H^+] + (k_1k_4k_5k_6k_7 + k_1k_4k_5k_6k_8 + k_2k_3k_4k_5k_6 + k_3k_4k_5k_6k_8)[H^+]^2\}/\text{denominator}.$$

Model I: Hydrogen Oxidation

$$\text{Denominator} = k_{-1}k_{-2}k_{-3}k_{-4}k_{-7} + k_{-1}k_2k_{-3}k_{-4}k_{-6}[H^+] + k_{-1}k_{-3}k_{-4}k_{-6}k_{-8}[H_2].$$

$$x_1 = \{k_{-1}k_{-2}k_{-3}k_{-5}k_{-7}k_{-8} + (k_{-1}k_{-2}k_5k_{-6}k_{-7}k_{-8} + k_{-2}k_{-3}k_{-4}k_{-5}k_7k_{-8})[H^+] + k_{-2}k_{-4}k_5k_{-6}k_7k_{-8}[H^+]^2\}[H_2]/\text{denominator}.$$

$$x_2 = \{k_{-1}k_{-2}k_{-3}k_{-4}k_{-5}k_{-8} + k_{-1}k_{-2}k_{-3}k_{-6}k_{-7}k_{-8} + (k_{-1}k_{-2}k_{-4}k_5k_{-6}k_{-8} + k_{-2}k_{-3}k_{-4}k_{-6}k_{-7}k_{-8})[H^+]\}[H_2]/\text{denominator}.$$

$$x_3 = k_{-1}k_{-2}k_{-3}k_{-4}k_{-6}k_{-8}[H_2]/\text{denominator}.$$

$$x_4 = \{(k_{-1}k_{-2}k_{-5}k_{-7}k_{-8} + k_{-2}k_{-3}k_{-5}k_{-7}k_{-8})[H_2] + (k_{-1}k_{-2}k_5k_7k_{-8} + k_{-2}k_{-3}k_{-5}k_7k_{-8} + k_{-2}k_{-4}k_{-5}k_7k_{-8} + k_{-2}k_5k_{-6}k_{-7}k_{-8})[H^+][H_2] + k_{-1}k_{-2}k_5k_{-7}k_8[H^+]^2 + (k_{-2}k_{-4}k_5k_7k_{-8} + k_{-2}k_5k_{-6}k_7k_{-8})[H^+]^2[H_2] + (k_{-1}k_2k_5k_{-7}k_8 + k_{-2}k_{-4}k_5k_7k_8)[H^+]^3 + k_2k_{-4}k_5k_7k_8[H^+]^4\}/\text{denominator}.$$

$$x_5 = \{(k_{-1}k_{-2}k_{-3}k_{-7}k_{-8} + k_{-1}k_{-2}k_{-4}k_{-5}k_{-8} + k_{-2}k_{-3}k_{-4}k_{-5}k_{-8} + k_{-2}k_{-3}k_{-6}k_{-7}k_{-8})[H_2] + (k_{-1}k_2k_{-3}k_{-5}k_{-7} + k_{-1}k_{-2}k_{-3}k_{-5}k_{-7} + k_{-1}k_{-2}k_{-3}k_{-7}k_8 + k_{-1}k_{-2}k_5k_{-6}k_{-7} + k_{-2}k_{-3}k_{-4}k_{-5}k_7)[H^+] + (k_{-1}k_{-2}k_{-4}k_5k_{-8} + k_{-1}k_{-2}k_5k_{-6}k_{-8} + k_{-2}k_{-3}k_{-4}k_7k_{-8} + k_{-2}k_{-3}k_{-6}k_7k_{-8} + k_{-3}k_{-4}k_{-5}k_7k_{-8})[H^+][H_2] + (k_{-1}k_2k_{-3}k_{-7}k_8 + k_{-1}k_2k_5k_{-6}k_{-7} + k_{-1}k_{-2}k_{-4}k_5k_8 + k_2k_{-3}k_{-4}k_{-5}k_7 + k_{-2}k_{-3}k_{-4}k_7k_8 + k_{-2}k_{-4}k_5k_{-6}k_7)[H^+]^2 + k_{-4}k_5k_{-6}k_7k_{-8}[H^+]^2[H_2] + (k_{-1}k_2k_{-3}k_5k_8 + k_2k_{-3}k_{-4}k_7k_8 + k_2k_{-4}k_5k_{-6}k_7)[H^+]^3\}/\text{denominator}.$$

$$x_6 = \{(k_{-1}k_{-2}k_{-3}k_{-4}k_{-5} + k_{-1}k_{-2}k_{-3}k_{-6}k_{-7} + (k_{-1}k_{-2}k_{-3}k_{-4}k_{-8} + k_{-1}k_{-2}k_{-3}k_{-6}k_{-8})[H_2] + (k_{-1}k_2k_{-3}k_{-4}k_{-5} + k_{-1}k_2k_{-3}k_{-6}k_{-7} + k_{-1}k_{-2}k_{-3}k_{-4}k_8 + k_{-1}k_{-2}k_{-4}k_5k_{-6} + k_{-2}k_{-3}k_{-4}k_{-6}k_7)[H^+] + (k_{-1}k_{-4}k_5k_{-6}k_{-8} + k_{-3}k_{-4}k_{-6}k_7k_{-8})[H^+][H_2] + (k_{-1}k_2k_{-3}k_{-4}k_8 + k_{-1}k_2k_{-4}k_5k_{-6} + k_2k_{-3}k_{-4}k_{-6}k_{-7})[H^+]^2\}/\text{denominator}.$$

Model II: Hydrogen Production

$$\text{Denominator} = k_1k_4k_5k_8(k_3k_5 + k_3k_{-7} + k_7k_9 + k_3k_7[H^+])[H^+].$$

$$x_1 = \{k_1k_{-2}k_3k_5k_{-6}k_7k_9 + (k_1k_{-2}k_3k_6k_7k_8k_9 + k_2k_3k_4k_5k_{-6}k_7k_9)[H^+] + k_2k_3k_4k_6k_7k_8k_9[H^+]^2\}[H^+]^2/\text{denominator}.$$

$$x_2 = k_1k_3k_9\{k_{-2}k_5k_7k_8 + k_4k_5k_{-6}k_7 + (k_4k_5k_7k_8 + k_4k_6k_7k_8)[H^+]\}[H^+]^2/\text{denominator}.$$

$$x_3 = k_1k_3k_4k_5k_7k_8k_9[H^+]^2/\text{denominator}.$$

$$x_4 = k_7k_9\{k_1k_{-2}k_{-3}k_{-6} + (k_1k_{-2}k_3k_{-6} + k_2k_{-3}k_4k_{-6} + k_{-2}k_3k_5k_{-6})[H^+] + (k_2k_3k_4k_{-6} + k_2k_3k_5k_{-6} + k_{-2}k_3k_6k_8)[H^+]^2 + (k_2k_3k_4k_6 + k_2k_3k_6k_8)[H^+]^3\}[H^+]/\text{denominator}.$$

$$x_5 = \{k_1k_{-2}k_{-3}k_5k_{-6}k_9 + k_1k_{-2}k_{-3}k_7k_8k_9 + k_1k_{-2}k_4k_{-6}k_7k_9 + k_1k_{-2}k_5k_{-6}k_7k_9 + k_1k_{-2}k_6k_7k_9 + (k_1k_2k_4k_6k_7k_9 + k_1k_{-2}k_3k_5k_{-6}k_7 + k_1k_{-2}k_3k_7k_8k_9 + k_1k_3k_4k_{-6}k_7k_9 + k_1k_3k_5k_{-6}k_7k_9 + k_1k_3k_6k_7k_8k_9 + k_2k_3k_4k_5k_{-6}k_{-7} + k_2k_3k_4k_5k_{-6}k_9 + k_2k_{-3}k_4k_7k_8k_9 + k_{-2}k_3k_5k_7k_8k_9 + k_3k_4k_5k_{-6}k_7k_9)[H^+] + (k_1k_{-2}k_3k_6k_7k_8 + k_1k_{-2}k_3k_6k_7k_9 + k_2k_3k_4k_5k_{-6}k_7 + k_2k_3k_4k_6k_{-7}k_9 + k_2k_3k_4k_7k_8k_9 + k_2k_3k_5k_7k_8k_9 + k_3k_4k_6k_7k_8k_9)[H^+]^2 + k_2k_3k_4k_6k_7k_8[H^+]^3\}[H^+]/\text{denominator}.$$

$$x_6 = \{k_1k_{-2}k_3k_5k_8k_9 + k_1k_{-2}k_4k_7k_8k_9 + k_1k_{-2}k_5k_7k_8k_9 + k_1k_3k_4k_5k_{-6}k_{-7} + k_1k_3k_4k_5k_{-6}k_9 + (k_1k_{-2}k_3k_5k_7k_8 + k_1k_3k_4k_5k_{-6}k_7 + k_1k_3k_4k_6k_{-7}k_8 + k_1k_3k_4k_7k_8k_9 + k_2k_3k_4k_5k_{-7}k_8 + k_2k_3k_4k_5k_8k_9 + k_3k_4k_5k_7k_8k_9)[H^+] + k_3k_4k_7k_8(k_1k_6 + k_2k_5)[H^+]^2\}[H^+]/\text{denominator}.$$

Model II: Hydrogen Oxidation

$$\text{Denominator} = k_{-1}k_{-4}k_{-5}k_{-8}(k_{-2}k_{-6} + k_2k_{-6}[H^+] + k_{-2}k_{-9}[H_2]).$$

$$x_1 = k_{-2}k_{-9}\{k_{-1}k_{-3}k_{-5}k_{-6}k_{-7} + (k_{-1}k_{-3}k_{-6}k_7k_{-8} + k_{-3}k_{-4}k_{-5}k_{-6}k_{-7})[H^+] + k_3k_{-4}k_{-6}k_7k_{-8}[H^+]^2\}[H_2]/\text{denominator}.$$

$$x_2 = k_{-2}k_{-6}k_{-9}\{k_{-1}k_{-3}k_{-5}k_{-8} + k_{-1}k_{-4}k_{-5}k_{-7} + (k_{-1}k_{-4}k_7k_{-8} + k_3k_{-4}k_{-5}k_{-8})[H^+]\}[H_2]/\text{denominator}.$$

$$x^3 = k_{-1}k_{-2}k_{-4}k_{-5}k_{-6}k_{-8}k_{-9}[H_2]/\text{denominator}.$$

$$x_4 = (k_{-2}k_{-3}k_{-6}k_{-7}k_{-9}(k_{-1} + k_{-5})[H_2] + \{k_{-1}k_{-2}k_{-3}k_{-6}k_7k_9 + k_{-2}k_{-6}k_{-9}(k_{-1}k_{-3}k_7 + k_3k_{-4}k_{-7} + k_3k_{-5}k_{-7} + k_{-3}k_7k_{-8})[H_2]\}[H^+] + \{k_{-6}k_7k_9(k_{-1}k_2k_{-3} + k_{-2}k_3k_{-4}) + k_{-2}k_3k_{-9}(k_{-4}k_{-6}k_7 + k_{-5}k_6k_{-7} + k_{-6}k_7k_{-8})[H_2]\}[H^+]^2 + (k_2k_3k_{-4}k_{-6}k_{-7}k_9 + k_{-2}k_3k_6k_7k_{-8}k_{-9}[H_2])[H^+]^3)/\text{denominator}.$$

$$x_5 = (k_{-2}k_{-3}k_{-5}k_{-6}(k_{-1}k_{-7} + k_{-1}k_9 + k_{-2}k_{-7}) + k_{-2}k_{-9}(k_{-1}k_{-3}k_{-5}k_{-6} + k_{-1}k_{-4}k_{-6}k_{-7} + k_{-1}k_{-5}k_6k_{-7} + k_{-1}k_{-5}k_{-6}k_{-7} + k_{-1}k_{-6}k_{-7}k_{-8} + k_{-3}k_{-5}k_{-6}k_{-8})[H_2] + \{k_{-1}k_2k_{-3}k_{-5}k_{-6}k_{-7} + k_{-1}k_2k_{-3}k_{-5}k_{-6}k_9 + k_{-1}k_{-2}k_{-3}k_{-6}k_7k_{-8} + k_{-1}k_{-2}k_{-4}k_{-6}k_7k_9 + k_{-2}k_3k_{-4}k_{-5}k_{-6}k_9 + k_{-2}k_{-3}k_{-4}k_{-6}k_7k_{-8} + k_{-9}(k_{-1}k_{-2}k_{-3}k_7k_{-8} + k_{-1}k_{-2}k_{-4}k_{-6}k_7 + k_{-1}k_{-2}k_{-5}k_6k_{-7} + k_{-2}k_3k_{-4}k_{-5}k_{-6} + k_{-2}k_3k_{-5}k_{-6}k_{-8} + k_3k_{-4}k_{-5}k_{-6}k_{-7})[H_2]\}[H^+] + \{k_2k_{-6}(k_{-1}k_{-3}k_7k_{-8} + k_{-1}k_{-4}k_7k_9 + k_3k_{-4}k_{-5}k_{-7} + k_3k_{-4}k_{-5}k_9) + k_{-8}k_{-9}(k_{-1}k_{-2}k_6k_7 + k_{-2}k_3k_{-4}k_7 + k_{-2}k_3k_{-5}k_6 + k_3k_{-4}k_{-6}k_7)[H_2]\}[H^+]^2 + k_2k_3k_{-4}k_{-6}k_7k_{-8}[H^+]^3)/\text{denominator}.$$

$$x_6 = (k_{-2}k_{-5}k_{-6}(k_{-1}k_{-3}k_{-8} + k_{-1}k_{-4}k_{-7} + k_{-1}k_{-4}k_9 + k_{-3}k_{-4}k_{-8}) + k_{-1}k_{-5}k_{-9}(k_{-2}k_{-3}k_{-8} + k_{-2}k_{-4}k_{-6} + k_{-2}k_{-6}k_{-8} + k_{-4}k_{-6}k_{-7})[H_2] + \{k_{-1}k_{-6}(k_2k_{-3}k_{-5}k_{-8} + k_2k_{-4}k_5k_9 + k_2k_{-4}k_{-5}k_{-6} + k_{-2}k_{-4}k_7k_{-8}) + k_{-8}k_{-9}(k_{-1}k_{-2}k_{-4}k_7 + k_{-1}k_{-2}k_{-5}k_6 + k_{-1}k_{-4}k_{-6}k_7 + k_{-2}k_3k_{-4}k_{-5})[H_2]\}[H^+] + k_2k_{-4}k_{-6}k_{-8}(k_{-1}k_7 + k_3k_{-5})[H^+]^2)/\text{denominator}.$$

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3. PROPERTIES OF HYDROGENASE OF *MEGASPHAERA ELSDENII*

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3.1. SUMMARY

The catalytic activities of *Megasphaera elsdenii* hydrogenase are stimulated by salts. The stimulation is due to the anion: the more chaotropic the anion, the greater the effect.

Dithionite-reduced and dye-oxidized preparations of hydrogenase are inactivated by reaction with oxygen. The inactivation of the reduced enzyme by excess oxygen follows pseudo-first-order kinetics; the reaction order for the oxidized enzyme has not been established. The rate of oxygen-inactivation is decreased by bovine serum albumin.

The hydrogen production activity decreases in the presence of dimethylsulphoxide and ethylene glycol. The hydrogen oxidation activity is stimulated by dimethylsulphoxide, and the activity remains linear with time at concentrations up to 50% (v/v). Above 70% dimethylsulphoxide the steady-state activity of hydrogenase is abolished for both types of activity.

The enzyme is more stable in a hydrogen atmosphere than in an argon atmosphere, and the oxidized enzyme is more stable than the reduced enzyme.

The enzyme is isolated in the presence of dithionite and it is therefore reduced. When the enzyme is oxidized by treatment with 2,6-dichloroindophenol or with (bi)sulphite, its activity increases by up to 65%; this activation is not reversed when the enzyme is re-reduced. The increase in activity is associated with a change of the redox potential of the incubation medium to a less negative value; half of the maximum activation occurs at -0.41 V.

The EPR spectrum of the dithionite-reduced hydrogenase resembles that of a reduced ferredoxin-type of spectrum with two 4Fe-4S clusters. The EPR spectrum of the oxidized enzyme is similar to that of *Chromatium* high-potential iron-sulphur protein. No redox potentials can be ascribed to these spectra since the redox system changes upon freezing to liquid helium temperatures.

3.2. INTRODUCTION

In recent years highly purified preparations of hydrogenases have been reported from a wide range of microorganisms including obligately anaerobic [1-7], anaerobic phototrophic [8-11], facultatively anaerobic [12,13], aerobic phototrophic [14] and obligately aerobic [15,16] bacteria. The hydrogenases of the obligately anaerobic bacteria are nearly all 12Fe-12S proteins [1,4-7] and in general they have much higher catalytic activities [1,4,6,7] than the enzymes isolated from the other microorganisms [8-16]. Hydrogenase from the rumen bacterium *Megasphaera elsdenii* is inactivated by oxygen and it is therefore purified under anaerobic conditions and in the presence of the strong reducing agent sodium dithionite [6,7]. As has been reported previously [6,7] the enzyme is greatly stimulated by certain salts. This paper describes a detailed examination of the oxygen-inactivation and of the salt effects it also describes the storage stability of the enzyme and the effect of oxidizing agents on the catalytic activity. The EPR spectra of the enzyme at several redox states are shown.

3.3. MATERIALS AND METHODS

3.3.1. Purification of hydrogenase

Hydrogenase was purified from *Megasphaera elsdenii* as described previously [7].

3.3.2. Assays for hydrogenase

Hydrogen oxidation was determined spectrophotometrically with 1 mM methyl or benzyl viologen as electron-acceptor and 2-mercaptoethanol and a vitamin B_{12} derivative as oxygen-scavenging system. Hydrogen production activity was routinely determined manometrically with dithionite-reduced methyl viologen (final concentration 1 mM) as electron donor, as described before [7]. Where indicated hy-

drogen production was determined gas chromatographically with a Pye Unicam GCD instrument fitted with a Molecular Sieve 5A column (150 x 0.4 cm) and a katharometer thermal conductivity detection device; argon was used as carrier gas. Assays in which hydrogen production was followed by gas chromatograph were carried out in 20 ml glass bottles fitted with a rubber septum. The reaction mixture was as used in the manometric measurements. The reaction mixture minus dithionite, methyl viologen and hydrogenase was added to the glass bottles and made anaerobic by five cycles of evacuation and filling with argon gas. After the final cycle, dithionite followed by hydrogenase was added by syringe, the bottles were equilibrated in a shaking water-bath (100-150 strokes.min^{-1}; 4 cm/stroke) at 30°C. The reaction was initiated shortly afterwards by adding methyl viologen reduced with dithionite (0.5 mM) to give a final concentration of 1 mM of the dye. At known intervals (2-4 min) 10-100 µl gas samples were withdrawn with a syringe and injected into the gas chromatograph.

3.3.3. Stimulation of the hydrogen oxidation or hydrogen production by salts

The effect of several salts on the hydrogen production and oxidation activity in the standard assays was determined. When hydrogen production or hydrogen consumption activity increases linearly with salt the change can be defined as the "molar salt stimulation coefficient", which is; "the increase in hydrogen production or consumption activity.(molar concentration salt)$^{-1}$.(mg hydrogenase)$^{-1}$.
Previously [7] it was shown that the increase in hydrogen oxidation activity is linear with the increase in salt concentration up to about 0.5 M salt. Above this salt concentration the increase in activity is no longer linear, probably due to a decreased solubility of hydrogen gas [17]. The molar salt stimulation coefficient was calculated from the linear part of the salt concentration/activity curve. Where necessary the pH of the salts used were adjusted to pH 8.

3.3.4. Inactivation of the hydrogenase by oxygen

Inactivation of hydrogenase by oxygen was performed in a magnetically stirred vessel with an oxygen electrode (Rank Brothers, Bottisham, Cambridge) at the bottom as described by Laane *et al.* [18]. The system was made gas tight with a rubber stopper through which reactants could be added and through which at intervals samples could be withdrawn by syringe. The standard incubation mixture

contained in a final volume of 6 ml at 30°C: 100 mM Tris-HCl pH 8, 0.05 mM methyl viologen (as redox indicator), 0.5 mM dithionite. The liquid phase was stirred continuously; the atmosphere was oxygen-free argon. After addition of the dithionite-reduced hydrogenase (final concentration 0.2 μM), air was added to the gas phase, so that after oxidation of the dithionite the oxygen concentration was brought to 10 μM.

When oxygen inactivation of the oxidized enzyme was determined, the dithionite-reduced hydrogenase and the dithionite present in the vessel were oxidized by addition of about 0.7 ml of an anaerobic solution (5 mM) of 2,6-dichloroindophenol (Cl_2Ind) until the colour in the vessel became slightly blue due to unreduced Cl_2Ind. After addition of Cl_2Ind, oxygen was added until the O_2 concentration in the solution reached 10 μM. Samples were withdrawn from the vessel at intervals and injected into 20 ml oxygen-free glass bottles which contained the mixture for the hydrogen production assay but without methyl viologen. The hydrogen production activity of each sample was determined gas chromatographically as described above. Control experiments showed that Cl_2Ind did not affect the hydrogen production activity in the standard assay. The dithionite-reduced hydrogenase and the Cl_2Ind-oxidized hydrogenase will be called respectively reduced and oxidized (D) hydrogenase.

3.3.5. Effects of dimethylsulphoxide and ethylene glycol on the hydrogenase activity

The effects of dimethylsulphoxide (Me_2SO) and ethylene glycol in the standard reaction mixtures on the hydrogen production and consumption activities were determined in 200 mM rather than in 75 mM Tris-HCl pH 8, in order to avoid pH changes during the reaction [7]. The effect of storing with Me_2SO on the production activity of the reduced and oxidized (D) enzyme was also determined. Storage of the reduced enzyme was performed in 100 mM Tris-HCl pH 8 and 1 mM dithionite under hydrogen; the oxidized (D) enzyme was stored in 100 mM Tris-HCl pH 8, while 2-mercaptoethanol and vitamin B_{12} derivative were used as the oxygen scavenging system and in this case the gas phase was argon; the hydrogenase concentration was 0.3 μM, the temperature was 30°C. Since Me_2SO inhibits the production activity (see Results) and Me_2SO was added together with the enzyme on storing the hydrogenase in Me_2SO, the percentage Me_2SO in each activity assay was brought to 15% Me_2SO (v/v) to cancel the inhibitory effect.

3.3.6. Effect of storage conditions on the hydrogenase

The enzyme (final concentration 0.3 μM) was stored anaerobically in 20 ml glass bottles fitted with a rubber septum at 4°C in 150 mM phosphate buffer pH 8 and 2.5 mM dithionite, the gas phase was either hydrogen or argon. To ensure anaerobiosis the glass bottles were stored in anaerobic chambers filled with respectively hydrogen or argon gas. The effects of albumin (1 mg/ml), sodium chloride (1 M) in the presence and absence of albumin and of oxidation of the enzyme with either ferricyanide or Cl_2Ind on the stability were determined.

Oxidation of the enzyme with either ferricyanide or Cl_2Ind was performed by addition of stoicheiometric amounts of anaerobic solutions (respectively 20 and 10 mM) towards the amount of dithionite. Oxidant was added directly after addition of hydrogenase so that the dithionite concentration could not be affected due to the hydrogen production activity of the enzyme with dithionite as sole electron-donor [7].

3.3.7. Effects of oxidants and the pH of preincubation on the hydrogenase activity

The effects of the pH of the incubation buffer and the effects of oxidants on the reduced enzyme were studied in detail. The incubation buffers used were 100 mM Tris-HCl buffer pH 9 and 150 mM phosphate buffer pH 5-8. The gas phase was in general argon, the effect of replacement of argon by hydrogen atmosphere was also determined at pH 8 and pH 6, the dithionite concentration was 2 mM, the enzyme concentration was 0.3 μM. Anaerobic solutions of Cl_2Ind (10 mM), ferricyanide (5 mM) and sulphite were used as oxidants. Within 15 min after addition of the enzyme, whether or not followed by oxidation, the production activity was determined. Spectrophotometrically it was observed that at pH 6 reduced hydrogenase is oxidized by addition of sulphite and reduced again by addition of dithionite, indicating that the hydrogenase obeys the (bi)sulphite-dithionite redox couple.

The effects of incubation and oxidation of the hydrogenase at several pH values were determined with the standard hydrogen production and hydrogen oxidation assays. Both types of activity were determined in 250 mM Tris-HCl buffer to ensure that the pH of the reaction mixtures did not change on addition of hydrogenase samples incubated at pH values other than 8.

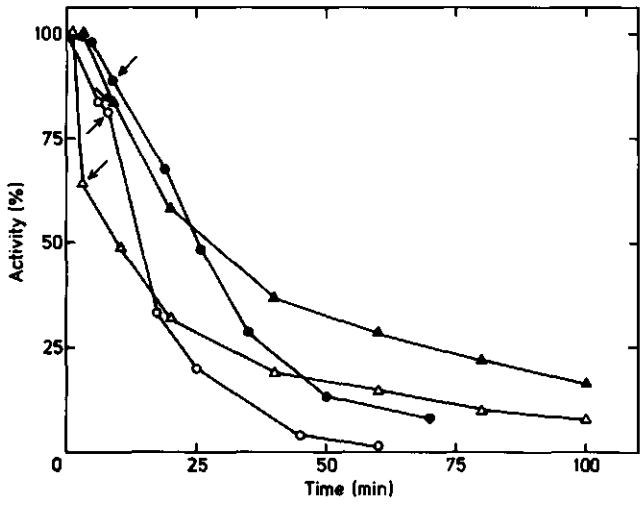

Figure 1. *Effects of oxygen on the activity of the dithiontie reduced and Cl_2Ind-oxidized enzyme.*
(o-o) inactivation of dihtionite-reduced hydrogenase by oxygen; (●-●) inactivation of dithionite-reduced hydrogenase by oxygen in the presence of albumin (1 mg/ml); (Δ-Δ) inactivation of the Cl_2Ind-oxidized hydrogenase by oxygen, (▲-▲) inactivation of the Cl_2Ind-oxidized hydrogenase by oxygen in the presence of albumin (1 mg/ml). The arrows indicate the time at which the O_2 concentration reached 10 μM, this oxygen concentration was maintained in time; the preceeding experimental point was the time at which O_2 was detected by the electrode. Activities are expressed as percentage of the activity before addition of oxygen and after addition of Cl_2Ind.

When Cl_2Ind is used to oxidize the dithionite and hydrogenase anaerobically, the activity in both the hydrogen production and hydrogen consumption assays increases by a factor of 1.55-1.65. When this oxidized (D) enzyme is subsequently treated with air it is inactivated (Fig. 1). The inactivation from the point at which the O_2 concentration reaches 10 μM ($t_{\frac{1}{2}}$ = 14 min) does not follow pseudo-first-order kinetics; the rate of inactivation also decreases in the presence of albumin ($t_{\frac{1}{2}}$ = 29 min). The changes caused by Cl_2Ind appear to be irreversible because neither the activity of the enzyme nor its rate of inactivation by O_2 are affected when the oxidized (D) enzyme is treated subsequently with dithionite. Control experiments show that addition of Cl_2Ind (final concentration 2 mM) to the reduced or oxidized (D) enzyme, after the oxygen concentration in the electrode vessel reaches 10 μM, does not affect the patterns of inactivation of the reduced and oxidized (D) enzyme, indicating that Cl_2Ind itself does not influence the inactivation.

3.4.3. *Effects of Me_2SO and ethylene glycol on the hydrogenase activity*

Since nearly all salts tested stimulate the catalytic activity of *M.elsdenii* hydrogenase (Table 1) the effect of decreasing the polarity in the assay mixture on the catalytic activity was studied. The effects of Me_2SO (v/v) and ethylene glycol (v/v) on the activities of the reduced and oxidized (D) enzyme in the standard hydrogen production assay are shown in Fig. 2A. At increasing percen-

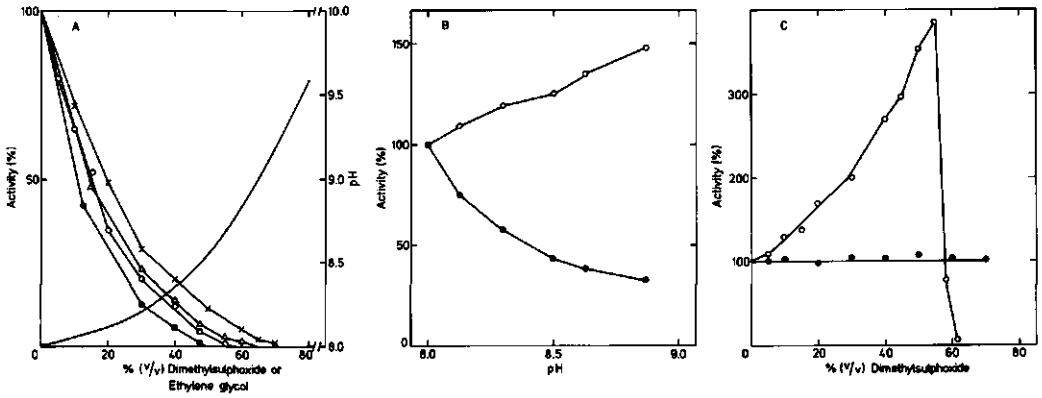

Figure 2. *Effects of Me_2SO, ethylene glycol and pH on hydrogenase activity.* (A) Effect of Me_2SO and ethylene glycol in the hydrogen production assay. (o-o) Effect of Me_2SO on the dithionite-reduced enzyme; (Δ-Δ) effect of Me_2SO on the Cl_2Ind-oxidized enzyme; (•-•) effect of Me_2SO on the dithionite-reduced and Cl_2Ind-oxidized enzyme in the presence of 1 M NaCl; (x-x) effect of ethylene glycol on the production activity of the dithionite-reduced and Cl_2Ind-oxidized enzyme in the absence or presence of 1 M NaCl; (——) effect of increasing percentages of Me_2SO on the pH of a 75 and 200 mM Tris-HCl buffer solution pH 8 (at 30°C) as measured by a glass-electrode. Activities are expressed as a percentage of the initial activity, without Me_2SO or ethylene glycol, in the absence or presence of NaCl. (B) Effect of pH on the (•-•) hydrogen production activity and (o-o) hydrogen oxidation activity with methyl viologen as electron acceptor. The Tris-HCl concentration was 200 mM. Activities are expressed as percentage of the activity at pH 8. (C) Effect of (o-o) Me_2SO and (•-•) ethylene glycol on the hydrogen oxidation activity. Activities are expressed as percentage of the activity without Me_2SO and ethylene glycol.

tages of Me_2SO the hydrogen production decreases. From 0-40% Me_2SO the activity is linear with time; at 47.5-55% Me_2SO a rapid decline in initial activity is followed by a constant activity, with both the reduced and oxidized (D) enzyme. At concentrations of Me_2SO higher than 60%, after a small initial activity, no hydrogen is produced by the reduced enzyme. The oxidized (D) enzyme is still able to produce hydrogen in 60% Me_2SO, at a constant rate. At concentrations of

Me_2SO higher than 65%, after a small initial activity, no hydrogen is produced by the oxidized (D) enzyme. The presence of NaCl (1 M) in the reaction mixtures enhances the effect of Me_2SO; the reduced and oxidized (D) enzyme show the same response to increasing Me_2SO in the presence of NaCl (Fig. 2A). The production activity is also decreased by ethylene glycol, the residual activity is still linear with time at 60% ethylene glycol; at 65-70% ethylene glycol a rapid decline in initial activity is followed by a constant activity. Sodium chloride (1 M) does not change the effect of ethylene glycol. The oxidized (D) and reduced enzyme show the same response to ethylene glycol in the absence and presence of NaCl (Fig. 2A). In contrast to the observation of Maurel *et al.* [19], who reported that 50% Me_2SO decreases the pH of a 10 mM Tris-HCl buffer pH 8.0 by 0.8, we observed that the pH, as measured by glass-electrode, of a 200 mM Tris-HCl buffer solution pH 8 becomes more alkaline upon the addition of Me_2SO (Fig. 2A) (cf. [20]). However, ethylene glycol has no pronounced effect on the pH. Since Me_2SO changes the pH (Fig. 2A), the effects of only the pH on the hydrogen production and consumption activities, in the absence of Me_2SO, was determined (Fig. 2B). The pH range tested was the same as that observed by addition of Me_2SO. The hydrogen oxidation activity with methyl viologen as electron acceptor is not affected by ethylene glycol up to 70%. Concentrations of Me_2SO up to 55%, however, increase the hydrogen oxidation activity; above 55% a very rapid decline in activity is observed (Fig. 2C). Above 50% Me_2SO the hydrogen oxidation activity is no longer linear in time; for example at 55%, 58%, 62% and 65% Me_2SO, after one minute the oxidation activity was respectively 36%, 25%, 10% and 0.4% of the initial activity. The reduced and oxidized (D) enzyme show similar responses to Me_2SO, but the specific activity of the oxidized (D) enzyme is about a factor 1.6-fold at each concentration of Me_2SO. The dependence of the hydrogen production and the hydrogen oxidation activities on the pH (Fig. 2B) in the absence of Me_2SO in the pH range induced by addition of Me_2SO differs from the effects caused by the pH changes induced by addition of Me_2SO (Fig. 2A,C).

The solubility of hydrogen increases with increasing concentrations of organic solvents [17]. This results in a higher substrate concentration and might explain the increase in hydrogen oxidation activity with increasing Me_2SO. However, if this were the explanation a similar increase might then have been expected for ethylene glycol. The absence of an increase in activity in ethylene glycol indicates that the stimulation in Me_2SO is due to effect(s) on the enzyme and is not simply the result of an enhanced substrate concentration.

The hydrogen oxidation activity with benzyl viologen as electron acceptor

could not be determined because at high concentrations of Me_2SO or ethylene glycol the colour of the semiquinone form changes from purple to blue, probably due to dissociation of a polymer of the reduced viologen (S.G. Mayhew, unpublished results).

The effect of storing the reduced and oxidized (D) enzyme anaerobically with Me_2SO is given in Table 2. Except for a small initial loss in activity the reduced enzyme is not affected by storing in Me_2SO up to 40%; above 45% Me_2SO

Table 2. *Effect of storing hydrogenase with Me_2SO.*
Hydrogen production activities were determined in 200 mM Tris-HCl buffer pH 8 with 1 mM methyl viologen semiquinone as electron donor and 50 mM dithionite as reductant. Activities are expressed as percentage of the activities on storing the dithionite-reduced and Cl_2Ind-oxidized enzyme in the absence of Me_2SO, after 0.25 h of incubation. The temperature during storage was 30°C.

Me_2SO	Activity of dithionite-reduced			Cl_2Ind-oxidized		
	stored for			stored for		
	0.25 h	6 h	24 h	0.25 h	6 h	24 h
% (v/v)	%					
0	100	100	100	100	100	100
15	103	100	100			
20				100	104	102
30	96	94	94			
40	93	91	91	100	98	99
45	90	87	80			
47.5				98	98	97
50	75	72	62			
55	17	14	6	48	45	46
60	0	0	0	11	8	3
65				2	0.1	0
70				0	0	0

the stability decreases. The oxidized (D) enzyme is almost unaffected by storing up to 47.5% Me_2SO; above 60% Me_2SO the enzyme inactivates rapidly. The stability of the enzyme under storage conditions (long-term effects; Table 2) differs sig-

nificantly from that of the enzyme under turn-over conditions (short-term effects; Fig. 2A,C).

3.4.4. Storage stability of the hydrogenase

As is shown in Table 3 the enzyme lost about 50% of its activity during 2 weeks of storage at 4°C under argon. The stability under argon is not improved by addition of albumin, NaCl, or NaCl plus albumin. Oxidation of the dithionite and reduced hydrogenase with either ferricyanide or Cl_2Ind in the presence of albumin tends to enhance the stability of the enzyme. Storage under hydrogen greatly increases the stability of the hydrogenase compared to storage under argon as gas phase. Addition of albumin increases the stability of the enzyme as was previously observed for *D. vulgaris* hydrogenase [4]. The most stable preparations are obtained by first treating the enzyme with just sufficient ferricyanide to oxidize excess dithionite, and then storing the enzyme under hydrogen. Further addition of ferricyanide (a 10^4 molar excess of un-reduced ferricyanide over hydrogenase) gave an immediate loss of 65% of the activity; the stability of this preparation is less than that of enzyme which has been treated with a stoicheiometric amount of ferricyanide towards dithionite. Oxidation with Cl_2Ind decreases the stability of the enzyme compared to oxidation with ferricyanide, with either argon or hydrogen as gas phase. The inactivation during storage under any condition cannot be described by a first-order process.

3.4.5. Effects of oxidants and the pH of preincubation on the hydrogenase activity

The enzyme is isolated at pH 8 in the presence of sodium dithionite. When the enzyme is pre-treated at a more acid pH value before assay, its activity increases (Table 4). The extent of the increase becomes greater with decreasing pH, and reaches 36% at pH 5. A change from pH 8 to pH 9 has little or no effect. The redox potential of the dithionite/(bi)sulphite system is known to become less negative with decreasing pH [22] and it was reasoned that the effect on the activity might be due to an associated change in the redox state of the enzyme. In support of this, an even greater activation is observed when the enzyme solution is pre-treated with (bi)sulphite to adjust the redox potential of the solution to less negative values. A similarly large activation can be achieved, even at pH 8, by treating the solution with sufficient Cl_2Ind to oxidize all of the di-

Table 3. *Effect of storage conditions on the stability of* M.elsdenii *hydrogenase.* Storage conditions were as described in Materials and Methods. Hydrogen production activities were performed in 75 mM Tris-HCl pH 8 and 1 M methyl viologen semiquinone as electron donor, as described previously [7]. Activities are expressed as percentage of the activity at zero time. a, 10^4 molar excess of ferricyanide over hydrogenase; the activity of this preparation was 35% of the activity of a preparation that had been treated with just sufficient ferricyanide to oxidize the dithionite. The hydrogenase concentration was 0.3 μM, the temperature during storage was 4°C.

Addition	Residual activity in gas phase					
	argon stored for (weeks)			hydrogen stored for (weeks)		
	2	4	9	2	9	20
	% initial					
None	46	14	0	97	91	77
albumin	55	12	0	98	95	87
NaCl	49	18	0			
albumin, NaCl	47	20	0			
albumin, $[Fe(CN)_6]^{3-}$	74	50	25	100	103	97
				81[a]	70[a]	15[a]
albumin, Cl_2Ind	39	31	14	65	35	13
NaCl, Cl_2Ind	2	1	0	66	37	4
albumin, NaCl, Cl_2Ind				83	57	12

Table 4. *Effects of oxidants and pH of preincubation buffer on the hydrogenase activity.*
Hydrogen production activities are expressed as a percentage of the activity of the enzyme incubated at pH 8 with argon as gas phase and were performed as described in Material and Methods. In expt 2, the Na_2SO_3 concentration was 150 mM; the pH of the stock solution of sodium sulphite used was adjusted to the pH of the incubation buffer with citric acid, the redox potentials of the (bi)sulphite/dithionite redox couple in the presence of 150 mM sulphite at pH 9, 8, 7, 6 and 5 are respectively -0.59, -0.48, -0.37, -0.30 and -0.23 V; the redox potentials in the absence of added sulphite are respectively -0.73, -0.62, -0.52, -0.44 and -0.37 V, the amount of sulphite in a 2 mM dithionite solution was determined to be 0.43 mM (values were calculated according to Mayhew [22]). In expts 3 and 4 $[Fe(CN)_6]^{3-}$ and Cl_2Ind were added in stoichiometric amounts to oxidize the dithionite. n.d. = not determined. Preincubation temperature 30°C, the gas phase during preincubation was either argon or hydrogen (as indicated).

Expt	Addition	Hydrogen production activity						
		argon at pH					hydrogen at pH	
		9	8	7	6	5	8	6
		%						
1.	-	96	100	109	124	136	97	126
2.	Na_2SO_3	97	107	132	156	165	112	162
3.	$[Fe(CN)_6]^{3-}$	n.d.	98	n.d.	113	n.d.	109	132
4.	Cl_2Ind	n.d.	154	n.d.	147	n.d.	165	158

thionite. Very little further increase occurs when the pH of Cl_2Ind-oxidized enzyme at pH 8 is changed to pH 6. The effects of pH, (bi)sulphite and Cl_2Ind are independent of the gas phase (H_2 or Ar) and are observed not only in the hydrogen production assay (Table 4) but also in hydrogen oxidation assays with methyl or benzyl viologen as electron acceptors (data not shown).

The activation cannot be reversed. When the pH of activated enzyme at pH 5 in the presence or absence of excess bisulphite is changed to pH 8 or 9, only a very small decrease in activity is observed (approx. 5%). Similarly, when fresh dithionite is added to Cl_2Ind-oxidized enzyme, the high activity remains.

A similar but smaller activation seems to occur when Cl_2Ind is replaced by $[Fe(CN)_6]^{3-}$. However, taking into account the effect of preincubation at different pH values (Table 4) addition of ferricyanide does not result in an enhanced activity.

3.4.6. EPR spectroscopy

Comparison of the EPR spectra of *M.elsdenii* hydrogenase at different redox states (Fig. 3) with EPR spectra of other non-heam iron proteins suggests that the spectrum of the reduced enzyme resembles that of an 8Fe-8S, "g = 1.94", ferredoxin-type of spectrum. The rhombic type of spectrum with the average g value above 2 (g_z = 2.101, g_y = 2.052 and g_x = 2.005) of the oxidized enzyme resembles the oxidized state of the Hipip (high-potential iron-sulphur protein) though the line shape is not typical of the Hipip-type. The spectra of the reduced and oxidized hydrogenase are very similar to the ones published for the hydrogenase of *C.pasteurianum* [2,21].

As has been shown by Mayhew [22], the concentrations of dithionite and (bi)-sulphite as well as the pH determine the redox potential of the dithionite/(bi)-sulphite redox couple and increasing dithionite concentrations cause the midpoint redox potential to become less negative. Williams-Smit *et al.* [23] have shown that, depending on the nature of the buffer, pH shifts of about 0-3 can be observed at sub-zero temperatures upon lowering the temperature. The occurrence of such a pH shift as well as oxidation due to an increased dithionite concentration are illustrated in Fig. 3. The reduced hydrogenase (Fig. 3A) was obtained in Tris-HCl buffer (pH 8.2 at 20°C). Tris-HCl buffer shifts to alkaline pH values upon freezing [23] and therefore the redox potential of the dithionite/(bi)sulphite redox couple becomes more negative, favouring the reduced state of the hydrogenase. In contrast it was observed that in phosphate buffer (pH 7.6), which becomes more acidic upon freezing, the enzyme is not completely reduced at liquid helium temperatures (Fig. 3B). On the other hand the visible absorption spectrum of this enzyme preparation showed that it was reduced at room temperature. The estimated pH shift upon freezing is about 1.8 and is clearly sufficient to change the redox potential in favour of the oxidized state. Further addition of dithionite results in an even more oxidized enzyme (Fig. 3C), in accordance with Mayhew [22]. EPR spectra of hydrogenase samples under identical circumstances to those described in Fig. 3B and 3C, but in the presence of albumin (15 $mg.ml^{-1}$) were the same; this observation does not agree with the proposal of William-Smith *et al.* [23]: they suggest that albumin tempers the pH shift due to freezing. Therefore we conclude that the pH values in the presence and absence of albumin are the same; however, the albumin concentration might not have been sufficient to prevent such a pH shift. In all cases shown here the enzyme preparations are kept under argon; replacement of argon by hydrogen in the case of

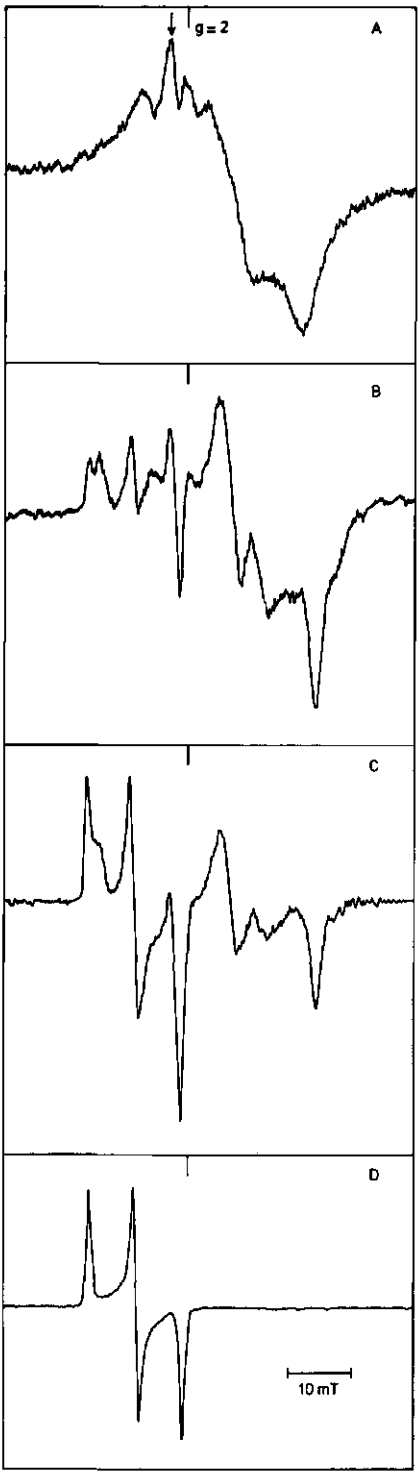

Figure 3. *EPR spectra of* M.elsdenii *hydrogenase at different redox states.* Spectrum A was obtained with hydrogenase (52 µM) in 100 mM Tris-HCl buffer pH 8 with 2 mM $Na_2S_2O_4$. EPR settings: microwave frequency, 9.098 GHz; microwave power, 125 µW; modulation amplitude, 0.8 mT; magnetic field sweep rate, 25 $mT.min^{-1}$; time constant, 0.3 sec; gain, 10^4; temperature 8.7 K. spectrum B was obtained with 52 µM hydrogenase in 100 mM phosphate buffer pH 7.6 with 2 mM $Na_2S_2O_4$. EPR settings: microwave frequency, 9.100 GHz; microwave power, 50 µW; modulation amplitude, 1 mT; magnetic field sweep, 12.5 $mT.min^{-1}$; time constant, 1 sec; gain, 2×10^4; temperature, 10 K. Spectrum C was obtained with the hydrogenase sample as described under spectrum B, the dithionite concentration was raised to 45 mM. EPR conditions are as for spectrum B. Spectrum D was obtained with hydrogenase (40 µM) in 100 mM phosphate buffer pH 6 with 1 mM $Na_2S_2O_4$ and 150 mM Na_2SO_3. EPR settings: microwave frequency, 9.084 GHz; microwave power 50 µW; modulation amplitude, 0.2 mT; magnetic field sweep, 2 $mT.min^{-1}$; time constant, 0.1 sec; gain, 10^4; temperature, 9.7 K. The modulation frequency was in all cases 100 kHz; all samples were under argon atmosphere.

the reduced enzyme does not change the features of the spectrum. An approximate integration of the signals (without field correction [24] and g-anisotropy) yielded values of about 0.6 and 1.2 electron respectively for the oxidized and reduced enzyme if compared with a copper standard solution (1 mM $CuSO_4$, 1 M $KClO_4$). Addition of 1 M KI had no marked effect on the features of the EPR spectra of either oxidized or reduced hydrogenase.

In Fig. 3A the peak indicated with an arrow shows a temperature and saturation behaviour that is different from the rest of the spectrum. This peak can be observed at temperatures up to 70 K, while the other absorptions have completely disappeared. A careful comparison shows that the peak is always present but that its intensity differs from preparation to preparation and can also be enhanced in several ways. For example, the addition of unbuffered anaerobic Tris solution (final concentration 130 mM) to enzyme in 100 mM phosphate pH 7.6 plus 45 mM dithionite (Fig. 3C) changes this EPR spectrum and gives the spectrum of the reduced enzyme (the pH became alkaline and causes the midpoint redox potential of the dithionite to become more negative); the intensity of the peak indicated increases. Control experiments show that the activity drops on addition of unbuffered Tris solution. The peak cannot be seen in the spectrum of the oxidized enzyme (Fig. 3D). Further oxidation of the oxidized enzyme with excess ferricyanide (final concentration about 0.5 mM) leads to a 50% increase in signal (about 1 electron equivalent) without a change in the features of the spectrum. However, the hydrogenase activity drops considerably to about 50% of the original activity. Further addition of ferricyanide (14 mM) shows the appearance of the peak described above. We conclude therefore that this peak has to be assigned to irreversibly inactivated enzyme. A similar peak has been observed for the hydrogenase in its reduced state as isolated from *D.vulgaris* [4] (H.J. Grande, unpublished results).

3.5. DISCUSSION

As is shown in Table 1 nearly all salts tested stimulate the catalytic activity of hydrogenase of *M.elsdenii*, in contrast to the hydrogenases of *D.vulgaris* [6] and *A.eutrophus* [15] which are inhibited by a high ionic strength. It is shown clearly that the main effect of stimulation is due to the anion. Similar effects have been observed with fumarase [25], D-glycerate dehydrogenase [26] and malate dehydrogenase [27] which are also stimulated by anions, though the activation might depend on the valency of the anion [25] and in these cases, in-

hibition is observed when the salt concentration exceeds 0.2 M.

With respect to the salt stimulation, the hydrogenase somewhat resembles proteins from halophilic bacteria, which are more or less dependent for their stability and activity on a salt concentration greater than 0.5 M NaCl [28]. In contrast to proteins from *Halobacteriacaea*, which are dependent on high ionic strength for their stability, *M.elsdenii* hydrogenase is stable at a relatively low ionic strength. As has been pointed out by Lanyi [28], an increase in enzymic activity that accompanies an increase in salt concentrations above 0.5 M, cannot be explained by electrostatic shielding. Above 0.5 M salt new hydrophobic interactions will appear, which are not stabilized in the absence of salt; these newly formed interactions might affect the activity.

It is noticeable, that for the hydrogenase activity the molar salt stimulation coefficient increases with increasing chaotropicity of the anion [28]. This indicates that the efficiency to disrupt the structure of water also contributes to the stimulation coefficient.

There are no pronounced changes in the features of the EPR spectra of the oxidized and reduced enzyme if salts are present which suggests that the stimulation by salts could be due to effect(s) on the protein structure and/or protein-Fe-S cluster(s) interaction(s) rather than on the Fe-S cluster(s) itself (themselves). Furthermore it is noteworthy that guanidine-HCl and Me_2SO inhibit the hydrogen production activity and stimulate the oxidation activity; these effects might indicate that the pathway(s) followed in hydrogen production differ(s) from that followed for hydrogen oxidation, as was suggested in kinetic models proposed earlier for the enzyme [7].

Recently it was reported by Klibanov *et al.* [30] that the partially purified hydrogenase of *C.pasteurianum* can also be protected against oxygen-inactivation by addition of albumin, as is the case for the *M.elsdenii* enzyme.
The difference in response to oxygen of the reduced and Cl_2Ind-oxidized enzyme, together with the increase in activity on oxidation indicates an irreversible change within the enzyme upon oxidation, which also leads to an enhanced stability against oxygen. It is somewhat surprising that in contrast to oxidation with Cl_2Ind and sulphite, neither oxidation with oxygen (Fig. 1) nor oxidation with ferricyanide (Table 4) increases the activity. A possible explanation for this difference could be the very positive midpoint potentials of these oxidants compared to those of Cl_2Ind and the (bi)sulphite/dithionite couple, leading to activation of part of the enzyme and inactivation of the rest by "over-oxidation". Control experiments with enzyme oxidized by either Cl_2Ind or sulphite

show no change in kinetic behaviour in the hydrogen production/substrate saturation curve with methyl viologen semiquinone as electron donor; the molar salt stimulation coefficients for NaCl and KI for the hydrogen production increase in proportion with the increase in activity due to oxidation.

The oxidized (*D*) enzyme is also more resistant against Me_2SO than the reduced hydrogenase (Fig. 2A, Table 2). Part of the decrease in production and increase in consumption activity at increasing percentages of Me_2SO can be explained by the alkaline pH shift of the Tris-HCl buffer by Me_2SO. As noted before, the increased hydrogen oxidation activity in Me_2SO cannot be explained by a higher substrate (hydrogen) concentration. Possible explanations for the effects of Me_2SO might be: (a) a change in protein structure; (b) a cathodic shift of the iron-sulphur clusters due to Me_2SO [31], although this shift is not very pronounced in up to 40% Me_2SO [31]; (c) changes in the values of the kinetic constants for the models, as described perviously [7]. Sodium chloride may intensify the effect(s) of Me_2SO on the hydrogenase. Ethylene glycol only affects the production activity; this might indicate that there is only a change in the values of the kinetic constants. Chen and Mortenson [32] have shown that hydrogenase of *C.pasteurianum* in 80% Me_2SO under hydrogen is able to reduce methyl viologen, but cannot reduce ferredoxin; in contrast, it cannot oxidize methyl viologen semiquinone, but can oxidize reduced ferredoxin. However, Chen and Mortenson did not state whether the observed catalytic activity in 80% Me_2SO was the initial, or the steady-state activity. In the case of *M.elsdenii* hydrogenase, after a small initial activity, no further activity is observed in 70% Me_2SO; all catalytic activity is also lost during 15 min preincubation in 70% Me_2SO. In this respect *M.elsdenii* hydrogenase more closely resembles *D.vulgaris* hydrogenase as isolated by van der Westen *et al.* [4] and *Chromatium vinosum* hydrogenase as isolated by van Heerikhuizen *et al.* [33]. The *D.vulgaris* enzyme does not show catalytic activity in 70% Me_2SO and loses all activity during preincubation in 70% Me_2SO (H.J. Grande, unpublished results). *Chromatium* hydrogenase does not lose activity when preincubated in 60% Me_2SO, but no activity was observed after preincubation in 80% Me_2SO; the hydrogen oxidation activity is also stimulated by Me_2SO, with a maximum stimulation at 20% Me_2SO, while no activity is observed at 60% Me_2SO (H. van Heerikhuizen, personal communication).

Storage under hydrogen is far more favourable than storage under argon (Table 3); the oxidized enzyme is more stable than the reduced enzyme. The observed difference in storage stability of the enzyme under argon and hydrogen might be explained by the fact that dithionite decomposes spontaneously and

autocatalytically, especially at acidic pH values [34]. The anaerobic decomposition products of dithionite may inactivate the *M.elsdenii* hydrogenase. Under a hydrogen atmosphere the decomposition products could be recycled by hydrogenase to form dithionite, a reaction that is not possible when the gas phase is argon. The addition of excess ferricyanide (see legend of Table 3) causes an immediate decrease of 65% in activity. The yellow colour due to the excess ferricyanide disappeared, indicating that hydrogenase is able to reduce ferricyanide. However, the stability of this preparation is lower than a preparation which has been treated with a stoicheiometric amount of ferricyanide. The use of pyruvate together with ferricyanide (fivefold molar excess of pyruvate over ferricyanide) to neutralize the possible hazardous effect on hydrogenase of a loosely bound CN^- in the hexacyanide complex [35], does not affect the results of experiments in which ferricyanide is used as oxidant.

As is shown in Table 4, preincubation of hydrogenase at acidic pH values increases the activity irreversibly; the increase in activity is proportional to the decrease in pH and independent of the gas phase. Oxidation of the hydrogenase with Cl_2Ind also increases the activity, independent of the pH of the preincubation buffer and gas phase. Sodium sulphite enhances the irreversible activation at decreasing pH values. Fig. 4 gives the relation between the redox potential of the (bi)sulphite/dithionite redox couple, calculated for each pH value of the incubation buffer, in the absence and presence of excess sodium sulphite and the irreversible activation at the corresponding potentials. It is clear that the irreversible activation depends on the redox potential of the incubation buffer and not on the pH. The potential at which half of the maximal activation is obtained is -0.41 V ($\pm$ 0.02 V). Apparently on changing the redox potential of the system to less negative values, the enzyme becomes irreversibly activated without appreciable changes in its kinetic properties. In our opinion the most probable explanation is that on oxidizing the hydrogenase the interactions between the protein part of the hydrogenase and the iron-sulphur clusters change, resulting in a higher rate of intermolecular electron and/or proton transport from the primary electron acceptors in the enzyme (Fe-S clusters) to the catalytic centre. This oxidized form of the enzyme is more stable to oxygen and Me_2SO and also has a higher storage stability. Further, the activation phenomenon follows the redox potential of the (bi)sulphite/dithionite couple rather than the hydrogen/proton couple, as is shown in Table 4. The question of whether the potentials of the iron-sulphur clusters are changed during this careful oxidation is being studied.

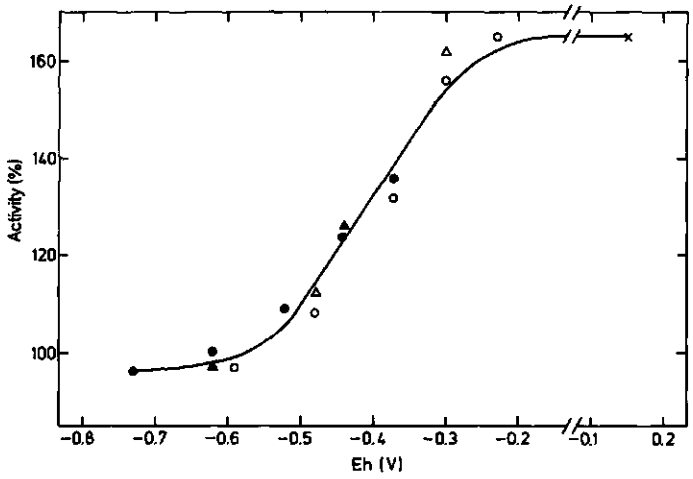

Figure 4. *Relationship between the (bi)sulphite/dithionite redox potentials of the hydrogenase incubation buffers and the irreversible increase in activity.* Redox potentials of the buffers were calculated according to Mayhew [21] (see legends of Table 4). (●) activities observed after preincubation at different pH values without added sulphite (gas phase, argon); (○) activities observed after preincubation at different pH values in the presence of 150 mM Na_2SO_3 (gas phase, argon); (▲) activities observed after preincubation at several pH values without added sulphite (gas phase, hydrogen). (△) activities observed after preincubation at several pH values in the presence of 150 mM Na_2SO_3 (gas phase, hydrogen); (x) maximal activity after oxidation with Cl_2Ind ($E_h \cong +0,15$ mV). Activities were measured in the hydrogen production assay and are expressed as percentage of the activity of hydrogenase preincubated at pH 8 without sulphite, with argon as gas phase.

The EPR spectra of the oxidized, reduced and intermediate redox states of the enzyme closely resemble the published EPR spectra of *C.pasteurianum* hydrogenase [2,21]. The spectrum of the reduced hydrogenase does not show proton splitting, favouring the kinetic model I (cf. [6]). For several reasons we are not able to ascribe redox potentials to the observed EPR spectra; firstly dithionite is contaminated by sulphite [22]; secondly, hydrogenase catalyses the oxidation of dithionite [7] thereby changing the (bi)sulphite/dithionite redox couple (the extent of the change before freezing is difficult to evaluate); thirdly, the pH of the buffer can shift drastically when samples are cooled to liquid nitrogen temperatures [23] leading to changes in the value of E' (the midpoint potential for the oxidation of SO_2^- [22]); and fourthly, it is not clear which value for temperature should be used in the Nernst equation. The

redox potential of the system evidently changes when the temperature is lowered and the change is presumably the result of an effect on the potential of the SO_2^-/HSO_3^- couple, which is known to become less negative with decreasing temperature [22,36], and partly the result of a pH change which also causes the SO_2^-/HSO_3^- potential to change, depending on the nature of the buffer used. It was recently reported that the midpoint potential of the SO_2^-/HSO_3^- couple changes by $-1.6\ mV.K^{-1}$ [22]; recalculation of the experimental data in Fig. 9 of that paper shows that this value is too low and that the correct value is $-2.2\ mV.K^{-1}$, a value in closer agreement with Jellinek's earlier value of $-2.26\ mV.K^{-1}$ [36].

Computer simulation of the spectrum of the oxidized hydrogenase shows that this spectrum can nicely be simulated (results not shown) with line widths of respectively 0.65, 0.55 and 0.65 mT according to a programme written by Scheppler [37]. Preliminary frequency-dependent EPR studies on the reduced hydrogenase show changes in the spectrum on changing the field, proving that there is some interaction between the clusters which give rise to the reduced ferredoxin-type signal.

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4. AN ANALYSIS OF ACTIVITY DETERMINATIONS IN A SERIES COUPLED REDOX REACTIONS WITH SPECIAL REFERENCE TO HYDROGENASE

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4.1. SUMMARY

The theoretical part of this paper gives the mathematical description of a system in which a redox mediator is continuously reduced by a redox system, in a non-rate limiting way and continuously enzymatically oxidized, in a rate-limiting way. Due to the changes in redox potential during the course of the reaction the ratio reduced/oxidized redox mediator declines gradually. It is possible to relate the changes in this ratio to the actual amount of product formed.

The product formation will be applied to the production of hydrogen by hydrogenase in a system with a redox mediator, which reduced form functions as ultimate electron donor for hydrogenase, and the dithionite/(bi)sulphite redox couple as reducing system. The changes in ratio reduced/oxidized redox mediator as function of pH and redox potential, followed spectrophotometrically, will be related to the hydrogen production activity of hydrogenase.

The second part of the paper deals with the analysis of the theory and describes the conditions under which the method can be used with methyl viologen as redox mediator.

The conditions to determine the pH dependence of the hydrogen production activity manometrically are also described.

4.2. INTRODUCTION

In general, hydrogenases catalyze the overall reaction

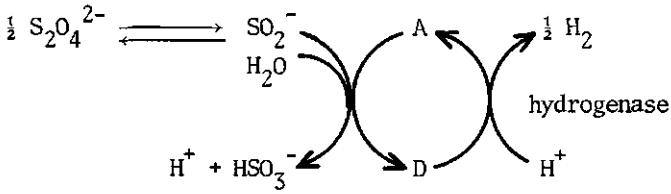
$$2\,H^+ + 2\,e \rightleftarrows H_2 \qquad (1)$$

The hydrogen evolution activity of hydrogenase is most commonly determined by following hydrogen formation in the presence of an electron carrier and an excess of dithionite ion to maintain a constant level of the reduced form of the electron carrier and a low redox potential. Since protons are substrate for hydrogenase the effect of pH on the production activity is of particular interest. As has been shown by Mayhew [1], however, the reducing capacity of dithionite is pH-dependent and decreases with decreasing pH, which results in a decreased concentration of reduced electron carrier at low pH values.

In this paper we describe the theoretical aspects and practical details of a spectrophotometric method to determine the hydrogen production activity as a function of pH and redox potential, as well as certain limitations of the manometric assay.

4.3. THEORY

The hydrogen production activity of hydrogenases is most commonly determined by measuring hydrogen evolution during the oxidation of a dithionite-reduced electron carrier [2]. Excess dithionite is required to maintain a constant level of reduced electron carrier and a low redox potential so that the amount of hydrogen produced is linear with time. The hydrogen produced is monitored manometrically, gas chromatographically or amperometrically. This redox mechanism is depicted in Scheme I.



Scheme I: *Hydrogen production from a dithionite-reduced electron carrier.* The dithionite/(bi)sulphite redox couple is in redox equilibrium with the D/A redox couple, where A is the oxidized and D the reduced form of the electron carrier. D functions as ultimate electron donor for hydrogenase. The dithionite concentration is higher than the concentration of reduced electron donor.

4.3.1. Mathematical description of Scheme I before addition of hydrogenase

Dithionite is in equilibrium with the SO_2^- radical which, according to Mayhew [1], functions as the reducing species. The concentrations of dithionite and (bi)sulphite determine the redox potential of the dithionite/(bi)sulphite

redox couple according to Eqn (2).

$$E_h = E' + \frac{RT}{F} \ln \frac{S_o(1 + \sqrt{1 + 8\,K_a S_r})}{2\,S_r} \qquad (2)$$

where E_h is the observed potential with reference to the hydrogen electrode at pH 0, and according to the notation of Mayhew [1] E' is the midpoint potential for the oxidation of the SO_2^- radical, which value is pH and temperature-dependent (cf. [1]). S_o and S_r are respectively the concentrations of the total oxidant and total reductant as described by Eqns (3-4); Eqns (5-6) describe the monomerisation of dithionite.

$$S_o = [HSO_3^-] + [SO_3^{2-}] \qquad (3)$$

$$S_r = [SO_2^-] + 2[S_2O_4^{2-}] \qquad (4)$$

$$[S_2O_4^{2-}] \underset{\leftarrow}{\xrightarrow{K_a}} 2[SO_2^-] \qquad (5)$$

$$K_a = [S_2O_4^{2-}]/[SO_2^-]^2 \qquad (6)$$

From Eqns (4) and (6):

$$S_r = 2[S_2O_4^{2-}] + \sqrt{[S_2O_4^{2-}]}/\sqrt{K_a} \qquad (7)$$

A value for the association constant of reaction 5 is: $K_a = 7.14 \times 10^8 M^{-1}$ [3].

According to Scheme I the dithionite/(bi)sulphite redox couple is in redox equilibrium with the reduced and oxidized forms of the electron carrier so it can be said that:

$$E_h = E' + \frac{RT}{F} \ln \frac{S_o\,(1 + \sqrt{1 + 8\,K_a S_r})}{2\,S_r} = E_1 + \frac{RT}{F} \ln \frac{[A]}{[D]} \qquad (8)$$

where E_1 is the midpoint potential of the electron carrier and A and D are respectively the concentrations of the oxidized and reduced forms of the electron carrier. If P is the concentration of the electron carrier added it follows that:

$$[A] = [P] - [D] \qquad (9)$$

Since E' and E_1 are, at a given pH value, constants a new constant K_1 can be defined:

$$K_1 = \exp(F(E_1 - E')/RT) \qquad (10)$$

Since the value of E' is pH-dependent [1], the value of K_1 decreases at decreasing pH values if the midpoint potential E_1 of the mediator is pH-independent (for example methyl viologen [1,4]). If E_1 is pH-dependent (for example *M.elsdenii* flavodoxin [5]; below pH 5.5 ΔE_1 is -59 mV per pH unit) the value of K_1 becomes constant below pH 5.5 since E' also changes with -59 mV/pH unit. Substituting Eqns (9-10) into Eqn (8), dividing both parts of the eqn by RT/F and leaving out the logarithm it follows that:

$$\frac{[P] - [D]}{[D]} = \frac{S_o(1 + \sqrt{1 + 8\ K_a S_r})}{2\ K_1 S_r} \tag{11}$$

Eqn (11) can be solved for [D]:

$$[D] = \frac{2[P]K_1 S_r}{2\ K_1 S_r + S_o(1 + \sqrt{1 + 8\ K_a S_r})} \tag{12}$$

Rewriting the square root term of the denominator of Eqn (12) by substituting Eqn (7) gives:

$$\sqrt{1 + 8\ K_a S_r} = \sqrt{1 + 16\ K_a[S_2O_4^{2-}]} + 2\sqrt{16\ K_a[S_2O_4^{2-}]} =$$

$$\sqrt{(1 + \sqrt{16\ K_a[S_2O_4^{2-}]})^2} = 1 + 4\ \sqrt{K_a[S_2O_4^{2-}]} \tag{13}$$

Substituting Eqns (7) and (13) into Eqn (12), followed by multiplication of the second term of the denominator by $([S_2O_4^{2-}]/K_a)/([S_2O_4^{2-}]/K_a)$ and next multiplication of the numerator and denominator by $\{\frac{1}{2}\sqrt{K_a}\ /(\frac{1}{2}(2[S_2O_4^{2-}] + \sqrt{[S_2O_4^{2-}]}/\sqrt{K_a})$ x $\sqrt{K_a})\}$ gives Eqn (14):

$$[D] = \frac{[P]\ K_1\sqrt{[S_2O_4^{2-}]}}{K_1\ \sqrt{[S_2O_4^{2-}]} + S_o\sqrt{K_a}} \tag{14}$$

4.3.2. Mathematical description of Scheme I after addition of hydrogenase

Addition of hydrogenase to the redox system as described in Scheme I results in the continuous oxidation of the dithionite-reduced electron donor D by hydrogenase to form hydrogen and the oxidized form of the electron donor A. The oxidized electron donor becomes continuously re-reduced by dithionite to form (bi)-sulphite and D. This cyclic process results in the ultimate oxidation of dithionite, mediated by an appropriate reduced electron donor (for example, methyl vio-

logen, ferredoxin, flavodoxin, cytc_3 [6-8]), to form stoicheiometric amounts of (bi)sulphite and hydrogen. Due to the oxidation of dithionite the dithionite/(bi)-sulphite redox couple becomes less negative, resulting in an identical change in redox potential of the D/A redox couple. However, this identical change in redox potential is only valid, when it is assumed that the rate of reduction of the oxidized electron donor by SO_2^- radical is faster than the rate of oxidation of the reduced electron donor by hydrogenase.

The increase in redox potential results in an increase in the concentration of A and a decrease in the concentration of D. It should be pointed out, that the changes in concentration of A and D do not equal, but only represent a measure of the amount of hydrogen produced. If the increase in concentration of A, or decrease in concentration of D can be measured as a function of time the decrease in dithionite concentration, which equals the amount of hydrogen produced can be derived in the following way:

The hydrogen production activity v is the amount of hydrogen produced per unit of time; since the amount of hydrogen produced equals the amount of dithionite oxidized it can be said that:

$$\frac{v}{[E]_t} = \frac{d\,H_2}{dt} = -\frac{d[S_2O_4^{2-}]}{dt} \tag{15}$$

Since the oxidation of each mole dithionite results in the formation of 2 moles (bi)sulphite it follows that:

$$-\frac{d[S_2O_4^{2-}]}{dt} = \tfrac{1}{2}\frac{d\,S_o}{dt} \quad \text{thus} \quad \frac{d\,S_o}{d[S_2O_4^{2-}]} = -2 \tag{16}$$

Measuring the decrease in concentration of D as function of time it can be said that:

$$-\frac{d\,[D]}{dt} = -\frac{d\,[S_2O_4^{2-}]}{dt} \cdot \frac{d\,[D]}{d\,[S_2O_4^{2-}]} \tag{17}$$

Substituting Eqn (15) into Eqn (17) gives:

$$\frac{v}{[E]_t} = -\frac{d\,[D]}{dt} \cdot \frac{d\,[S_2O_4^{2-}]}{d\,[D]} \tag{18}$$

On the other hand, measuring the increase in concentration of A as function of time it can be shown in the same way that:

Table 1. *Calculated relation between pH, E_h, dithionite, (bi)sulphite and methyl viologen concentrations to obtain 0.3 mM of the methyl viologen in its semiquinone form.*
Calculations were performed according to Mayhew [1]. P is methyl viologen. It was assumed that the temperature was 30°C; the changes in midpoint potentials (-2.2 mV.K^{-1} for the dithionite/(bi)sulphite redox couple [10] and -0.6 mV.K^{-1} for methyl viologen [9]) due to the change in temperature from the value of 25°C used previously was taken into account.

Conditions		[P]	(bi)sulphite in mM at		
	E_h	total methyl viologen	$[S_2O_4^{2-}]$	$[S_2O_4^{2-}]$	$[S_2O_4^{2-}]$
	mV	mM	1 mM	2.5 mM	7.5 mM
pH 7.5					
E' = -724 mV					
E'_1 = -449 mV					
K_1 = 37520	-500	0.343	6.30	9.95	17.2
	-450	0.589	42.7	67.6	117
	-400	2.26	290	459	794
pH 6.5					
E' = -631 mV					
E'_1 = -449 mV					
K_1 = 1065	-450	0.589	1.21	1.92	3.32
	-400	2.26	8.23	13.2	22.5
	-350	13.6	55.9	88.3	153
pH 5.5					
E' = -563 mV					
E'_1 = -449 mV					
K_1 = 78.75	-400	2.26	0.61	0.96	1.67
	-350	13.6	4.13	6.53	11.3
	-300	906	28.0	44.3	76.8

Table 2. *Calculated values for $d[S_2O_4^{2-}]/d[D]$ for the conditions as described in Table 1 before (t=0) and after (t=t) addition of hydrogenase.*
Experimental conditions: an anaerobic cuvette (0.4 cm light path) contained at t=0, 0.3 mM methyl viologen semiquinone (ε_{604} = 13.6 $mM^{-1}cm^{-1}$ [1]). Values for $d[S_2O_4^{2-}]/d[D]$ at t=0 are given in columns 3-5. After addition of hydrogenase and a decrease in A_{604} of 0.05 (t=t), corresponding with a decrease in the concentration of methyl viologen semiquinone of 9.2 µM, values for $d[S_2O_4^{2-}]/d[D]$ were recalculated and are expressed as percentage of the corresponding value of $d[S_2O_4^{2-}]/d[D]$ at t=0 (columns 6-8).

	E_h	$d[S_2O_4^{2-}]/d[D]$ at t=0			$d[S_2O_4^{2-}]/d[D]$ at t=t expressed as % at t=0		
		$[S_2O_4^{2-}]$ at t=0			$[S_2O_4^{2-}]$ at t=0		
	mV	in mM			in mM		
		1	2.5	7.5	1	2.5	7.5
pH 7.5							
K_1 = 37520							
	-500	32.8	66.9	146.9	74	77	90
	-450	12.4	29.6	81.1	90	91	93
	-400	7.58	18.8	55.6	96	96	96
pH 6.5							
K_1 = 1065							
	-450	3.16	5.47	10.2	102	103	104
	-400	5.17	10.9	24.7	100	100	012
	-350	6.36	15.3	42.7	98	99	100
pH 5.5							
K_1 = 78.75							
	-400	1.02	1.69	3.04	105	105	106
	-350	3.46	6.73	14.0	102	103	104
	-300	5.85	13.6	26.1	99	100	100

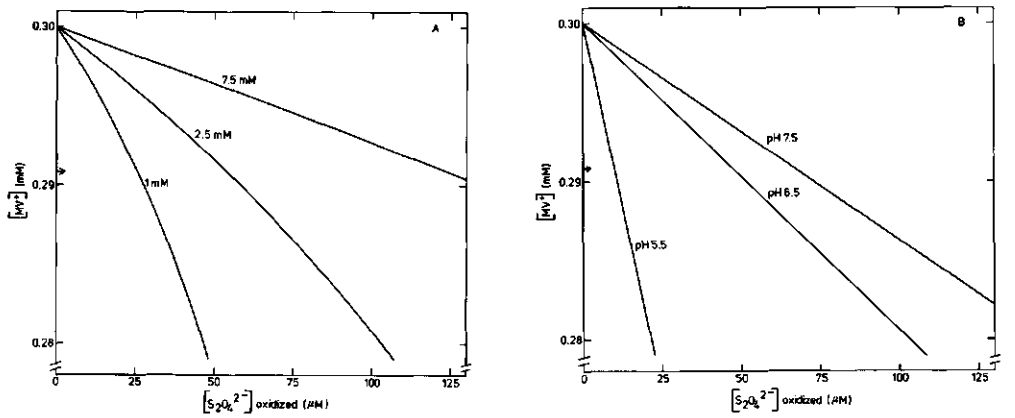


Figure 1. *Calculated relation between the concentration of methyl viologen semiquinone and the concentration of dithionite oxidized.*
(A) Calculated relation as function of the dithionite concentration, at constant pH and identical E_h. The experimental conditions assumed were as described in the legends of Table 1 and 2. The pH is 7.5; the initial value for E_h = -500 mV. The initial dithionite concentrations are indicated on each curve. The arrow indicates the point at which the decrease in absorbance is 0.05. (B) Calculated relation as function of pH, at the same initial dithionite concentration and identical E_h. The initial dithionite concentration is 1 mM, the initial value for E_h = -400 mV. The pH values are indicated on each curve. The arrow indicates the point at which the decrease in absorbance is 0.05.

concentration of dithionite that has to be oxidized to obtain the same decrease in methyl viologen semiquinone absorbance (Fig. 1A), indicating that -d[D]/dt is greater at low than at high dithionite concentration. In order to obtain the same value for v the correction factor at high dithionite concentrations has to be greater than at low dithionite concentrations. Secondly, the value of $d[S_2O_4^{2-}]/d[D]$ at a given redox potential and dithionite concentration decreases with decreasing pH. This is due to the fact that at lower pH values less dithionite has to be oxidized to obtain the same decrease in concentration of methyl viologen semiquinone, as is shown in Fig. 1B. Thus at pH < 6.5 the value of $d[S_2O_4^{2-}]/d[D]$ does not vary by more than 6% of its initial value; in contrast, the value at pH 7.5 can vary by up to 26%. Further it is noteworthy that at pH 7.5 the value of $d[S_2O_4^{2-}]/d[D]$ decreases as the redox potential becomes less negative while at pH 6.5 and pH 5.5 the opposite occurs. This can be explained in the following way: according to Mayhew [1], the effective midpoint potential, E_m, of the dithionite/(bi)sulphite couple is:

$$E_m = E' + \frac{RT}{2F} \ln 4K_a[S_2O_4^{2-}] \qquad (23)$$

It can be shown that if the effective midpoint potential is more negative than the midpoint potential of the electron donor the value of $d[S_2O_4^{2-}]/d[D]$ increases with increasing values for E_h, while the opposite occurs if the effective midpoint potential is less negative than that of the electron donor.

A change in the value of $d[S_2O_4^{2-}]/d[D]$ during the progress of the hydrogen production reaction implies, that the progress curve for the decrease of D is non-linear; such non-linearity is especially marked at high pH values (see Table 2). Since the tangent is drawn to the initial stage of the progress curve it is reasonable to assume that in practice the tangent is drawn through the points at t=0 and t=t; at t=t 9.2 μM methyl viologen semiquinone has been oxidized. If the values of $d[S_2O_4^{2-}]/d[D]$ for pH 7.5 and E_h = -500 mV are calculated according to the lines which connect the change in absorption in going from t=0 to t=t these values are 28.0, 59.4 and 136.3 for 1.0, 2.5 and 7.5 mM $[S_2O_4^{2-}]$ respectively; the values are 14, 11 and 7% less than the comparable values at t=0. This indicates that the redox system as described is also applicable at higher pH values though the confidence increases at decreasing pH values and becomes maximal below pH 7. However, from Table 2 it can be derived, that at pH 7.5, especially at low dithionite concentrations, the value of $d[S_2O_4^{2-}]/d[D]$ decreases, indicating that the activity will be slightly overestimated.
Thus the method described here can be used, firstly, to measure the hydrogen production activity of the hydrogenase at pH values below pH 7 and secondly, to study the effect of the redox potential on the hydrogenase activity.

4.4.2. Conditions that -d[D]/dt is constant

If it is assumed that the hydrogenase obeys Michaelis-Menten kinetics for the hydrogen production, the activity as a function of the electron donor concentration is given by:

$$\frac{v}{[E]t} = \frac{[D]\ V}{[E]t\ ([D] + Km)} \qquad (24)$$

where V is the maximal activity at infinite electron donor concentration and Km is the Km value for the electron donor at a given pH. According to Eqn (15)

$v = -d[S_2O_4^{2-}]/dt$. Substituting Eqn (24) into Eqn (18) it follows that:

$$\frac{[D]\ V}{[E]t\ ([D]+Km)} = -\frac{d[D]}{dt} \cdot \frac{d[S_2O_4^{2-}]}{d[D]} \tag{25}$$

Rewriting Eqn (25) gives:

$$\frac{[E]t\ ([D] + Km)}{[D]\ V}\, d[D] = -\frac{d[D]}{d[S_2O_4^{2-}]}\, dt \tag{26}$$

Since it has been shown in Table 2 that, in the first approximation, $-d[S_2O_4^{2-}]/d[D]$ can be assumed to be constant during the initial stage of the reaction, integration of both parts of Eqn (26) gives:

$$\frac{[E]t\ ([D] - [D_o])}{V} + \frac{[E]t\ Km}{V}\ (\ln[D] - \ln[D_o]) = -\frac{d[D]}{d[S_2O_4^{2-}]} .t \tag{27}$$

where D_o and D are the concentrations of the reduced electron donor at t=0 and t=t respectively. Multiplication of both parts of Eqn (27) with $V/Km[E]t$ and solving it for D gives:

$$\frac{[D]}{Km} + \ln [D] = \frac{[D_o]}{Km} + \ln [D_o] - \frac{V}{[E]t\ Km} \cdot \frac{d[D]}{d[S_2O_4^{2-}]} \cdot t \tag{28}$$

Rewriting ln [D] gives:

$$\ln [D] = \ln ([D] + [D_o] - [D_o] = \ln \left(\frac{[D] - [D_o]}{[D_o]} + 1\right) [D_o] =$$

$$= \ln \left(\frac{[D] - [D_o]}{[D_o]} + 1\right) + \ln [D_o] \tag{29}$$

Expansion of the logarithmic function in [D] of Eqn (29) gives:

$$\ln \left(\frac{[D] - [D_o]}{[D_o]} + 1\right) = \frac{[D] - [D_o]}{[D_o]} - \frac{1}{2}\left(\frac{[D] - [D_o]}{[D_o]}\right)^2 + \frac{1}{3}\left(\frac{[D] - [D_o]}{[D_o]}\right)^3 - \ldots \tag{30}$$

It can be shown that for [D] = 0.97 [D_o], corresponding to a decrease in absorbance of 0.05, the contribution of the terms with power greater that one only is 1.5% of the value of the term with power one, therefore neglectable. Substituting Eqn (30) into Eqn (28) and taking ln [D_o] of Eqn (27) into account:

$$\frac{[D]}{Km} + \frac{[D] - [D_o]}{[D_o]} + \ln [D_o] = \frac{[D_o]}{Km} + \ln [D_o] - \frac{V}{[E]t\ Km} \cdot \frac{d[D]}{d[S_2O_4^{2-}]} \cdot t \qquad (31)$$

Subtracting ln [D_o] from both parts of Eqn (31) and solving it for [D] gives:

$$[D] = [D_o] - \frac{[D_o]\ V}{[E]t\ ([D_o] + Km)} \cdot \frac{d[D]}{d[S_2O_4^{2-}]} \cdot t \qquad (32)$$

Eqn (32) predicts that the decrease in [D] as function of time is linear. In other words, -d[D]/dt is constant. Since both -d[D]/dt and $d[S_2O_4^{2-}]/d[D]$ are constant for the conditions described, $v/[E]t$ has to be constant.
It is of interest that if [D_o]<<Km Eqn (32) becomes:

$$[D] = [D_o] - \frac{[D_o]\ V}{[E]t\ Km} \cdot \frac{d[D]}{d[S_2O_4^{2-}]} \cdot t \qquad (33)$$

indicating that:

$$-\frac{d[D]}{dt} = -\frac{[D_o]\ V}{[E]t\ Km} \cdot \frac{d[D]}{d[S_2O_4^{2-}]} \qquad (34)$$

which predicts that the tangent drawn to the activity curve represents a measure of $[D_o]V/Km[E]t$ which gives a value for the ratio V/Km.
Eqn (33) can also be derived in an independent way for D_o<< Km; in this case the Michaelis-Menten eqn becomes $\frac{v}{[E]t} = \frac{[D_o]\ V}{[E]t\ Km}$.

4.4.3. Application to M.elsdenii *hydrogenase*

It can be shown in a way similar to that described above, that for the general rate equation for the hydrogen production activity of *M.elsdenii* hydrogenase, which is (cf. [6]):

$$\frac{v}{[E]t} = \frac{x_1[D] + x_2[D]^2 + x_3[D]^3}{x_4 + x_5[D] + x_6[D]^2 + [D]^3} \qquad (35)$$

that the decrease in concentration of D at the *initial* stage of the reaction is also constant. Thus the method described above to determine the effect of pH and redox potential on hydrogenase activity is also applicable for *M.elsdenii* hydrogenase.

4.5 EFFECT OF PH ON THE MANOMETRICALLY DETERMINED HYDROGEN PRODUCTION ACTIVITY

As is depicted in Scheme I due to the action of hydrogenase the dithionite/(bi)sulphite redox couple becomes less negative and causes the concentration of the reduced form of the electron donor to decrease. However, at sufficient high pH (pH>7.5) and dithionite concentration ($[S_2O_4^{2-}]$>10 mM) despite of this increase in redox potential, the dithionite/(bi)sulphite redox couple maintains such a low redox potential that the commonly used electron donors will be in their reduced forms. According to Mayhew [1], the redox potential of the dithionite/-(bi)sulphite redox couple is:

$$E_h = E' + \frac{RT}{F} \ln \frac{S_o}{[S_2O_4^{2-}]} + \frac{RT}{2F} \ln 4[S_2O_4^{2-}]K_a \qquad (36)$$

As can be derived from Eqn (36), when the ratio $S_o/[S_2O_4^{2-}]$ is small, oxidation of dithionite causes this ratio greatly to change, resulting in a relative large increase in E_h and a concomitant decrease in the concentration of the reduced electron donor. Addition of (bi)sulphite increases this ratio but also causes a smaller change in this ratio when dithionite becomes oxidized; thus the change in E_h is smaller.
An increase in volume of the reaction mixture in the Warburg vessel results per mole hydrogen produced in a smaller change of the dithionite concentration, thus a smaller change in E_h. The effects of addition of (bi)sulphite and enlargement of the reaction volume on the changes in concentration of the reduced electron donor (methyl viologen semiquinone) due to oxidation of dithionite by hydrogenase are shown in Table 3. As can be concluded from this table, it is recommended to use a reaction volume of 6 ml rather than of 2 ml in order to avoid drastic changes in the concentration of methyl viologen semiquinone and E_h, especially at pH values lower than pH 7.5.

Table 3. *Calculated change in the concentration of methyl viologen semiquinone as function of the amount of hydrogen produced at several pH values and dithionite/(bi)sulphite ratio's, and its dependence on the reaction volume in the manometric assay.*
The initial methyl viologen semiquinone concentration is 0.3 mM, the temperature is 30°C; a, dithionite concentration in the hydrogen production assay as used by van Dijk *et al.* [6]; b, dithionite concentration in the hydrogen production assay as used by Chen and Mortenson [11] and by van der Westen *et al.* [8]; c, (bi)sulphite contamination assumed to be present in dithionite solutions (see following paper).

					Reaction volume (ml)			
					2		6	
pH	$[S_2O_4^{2-}]$	S_o	E_h	$[MV^{2+}]$	amount of H_2 produced (µmol)			
					1.5	3	1.5	3
	mM	mM	mV	mM	$[MV^+]$ in µM after 1.5 or 3 µmol H_2 produced			
7.5	50[a]	11.1[c]	-536	311	298	297	300	299
	25	5.7[c]	-545	308	298	296	300	299
	25	10	-530	314	298	297	300	299
	15[b]	3.6[c]	-550	306	297	294	299	298
6.5	50	11.1	-443	673	278	258	292	295
	25	5.7	-452	572	265	236	287	276
	25	10	-437	777	272	248	290	281
	15	3.6	-457	522	252	215	282	266
5.5	50	11.1	-375	5350	261	233	287	275
	25	5.7	-384	3970	238	196	276	256
	25	10	-369	6750	259	227	285	272
	15	3.6	-389	3290	212	162	264	236

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5. THE EFFECTS OF PH AND REDOX POTENTIAL ON THE HYDROGEN PRODUCTION ACTIVITY OF THE HYDROGENASE FROM *MEGASPHAERA ELSDENII*

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5.1. SUMMARY

The effects of temperature on the ionization constant and apparent midpoint potential of the unprotonated species of *M.elsdenii* flavodoxin hydroquinone shows that above 15°C $\Delta pK'.K^{-1} = -9.7 \times 10^{-3}$ and $\Delta E_B.K^{-1} = -0.6$ mV.
The effects of pH and redox potential on the hydrogen production activity with methyl viologen semiquinone (0.3 mM; artificial donor) and *M.elsdenii* flavodoxin hydroquinone (50 µM; natural donor) show that at decreasing pH the activity increases.
Irrespective of the pH, at increasing redox potential, a redox potential-independent production activity is followed by a redox potential-dependent production activity. This redox potential-dependent behaviour of the hydrogen production activity represents a n=2 type redox titration curve with an 'apparent midpoint potential' which corresponds with the potential of the hydrogen electrode at that pH.
The effect of pH on the manometrically determined hydrogen production activity (direct) is in good agreement with that determined spectrophotometrically (indirect; see chapter 4 of this thesis), with both electron donors tested.

In contrast to predictions from the models for hydrogenase activity (van Dijk *et al.*, Eur.J.Biochem. *102*, 317-330) a double reciprocal plot of the kinetic data for *M.elsdenii* flavodoxin at pH 5.5 is non-linear. A slightly adapted kinetic model based on a similar mathematical formulation of its rate equation, to explain the effects of redox potential, proton and electron (donor) concentration on the hydrogenase activity is proposed. This model also explains, on a theoretical basis, the effects of pH and redox potential on the hydrogen production activity.
The effect of pH on the hydrogen oxidation activity with methyl and benzyl viologen as electron acceptors shows for both dyes an optimum at pH 9.7. The ratio of

the activities with methyl and benzyl viologen is constant over the pH range tested.

5.2. INTRODUCTION

The enzyme hydrogenase (E.C. 1.18), which catalyses the reversible activation of molecular hydrogen, is widespread in bacteria and algae [1]. To date many highly purified preparations of hydrogenase have been reported from a wide range of microorganisms [2-19]. Since hydrogenases are redox enzymes which catalyze the overall reaction:

$$2H^+ + 2e \rightleftarrows H_2 \qquad (1)$$

the effects of redox potential and the proton and electron (donor) concentration on the activity are of particular interest. As has been pointed out previously [20], studying the effect of the concentration of a dithionite-reduced electron donor above pH 7.5 is in general no problem. However, due to the effect of pH on the reducing capacity of dithionite [21], hydrogen production activity measurements at pH values below pH 7.5 have to be corrected for the effect of pH on the dithionite redox potential [20]. In our opinion the pH-dependent hydrogen production activity measurements as published by Erbes and Burris [22], Adams and Hall [12,19] and Arp and Burris [16] are not performed correctly since in all these studies the effect of pH on the reducing capacity of dithionite was not taken into account.

In this paper we present the results of a study of the effects of pH and redox potential on the hydrogen production activity, as determined spectrophotometrically [20] and manometrically, as well as the kinetics with *M.elsdenii* flavodoxin at pH 5.5. The effect of pH on the hydrogen oxidation activity was also studied.

5.3. MATERIAL AND METHODS

5.3.1. Purification of enzymes

Hydrogenase from *Megasphaera elsdenii* was purified as reported [17]. Flavodoxin from *M.elsdenii* was purified as described before [23]. The concentration of flavodoxin was determined from absorbance measurements at 445 nm (ε_{445} = 10.2 $mM^{-1}.cm^{-1}$).

5.3.2. Effect of pH and redox potential on the hydrogen production activity

As has been shown by Mayhew [21], the concentrations of dithionite and (bi)-sulphite, as well as the pH and the temperature determine the redox potential of the dithionite/(bi)sulphite redox couple. A decreasing pH and temperature, as well as increasing dithionite concentrations cause the effective midpoint potential to become less negative, resulting in a decrease in reducing capacity of dithionite. It is necessary to take these factors into account if the hydrogen production activity of the enzyme is determined at pH values below pH 7.5. At pH values below pH 7.5 the concentrations of the electron carriers (methyl viologen or *M.elsdenii* flavodoxin) and (bi)sulphite were calculated according to Mayhew [21], taking into account the effects on the redox potential of the pH, dithionite concentration, and temperature, so that the concentrations of methyl viologen semiquinone and flavodoxin hydroquinone were respectively 0.3 mM and 50 μM. If not otherwise stated these concentrations of reduced electron carriers were used throughout to study the effects of pH and redox potential on the hydrogen production activity of the enzyme. The pH-dependence of the hydrogen production activity was determined manometrically as well as spectrophotometrically.

For all activity measurements described below, the temperature was 30°C, the gas phase was argon for measurements of hydrogen production, or hydrogen when hydrogen oxidation activity was determined. The dithionite and sodium (bi)sulphite concentrations are given after reduction of the electron carrier; in all cases the contamination of dithionite (from Merck) by sodium(bi)sulphite, was taken into account. This contamination of dithionite by (bi)sulphite was determined spectrophotometrically by adding increments of a known dithionite solution to anaerobic solutions at pH 6.5-5.5 either containing *M.elsdenii* flavodoxin or methyl viologen at 25°C. From the redox potentials as indicated by the oxidized methyl viologen/semiquinone or the flavodoxin semiquionone/hydroquinone redox couples the (bi)-sulphite concentrations could be calculated according to Mayhew [1]. The average of eight determinations showed that our batch of dithionite contained 22% ± 2% (bi)sulphite. The dithionite and sodium(bi)sulphite solutions used were prepared freshly every day. The pH of the (bi)sulphite and methyl viologen solutions used, were adjusted to the pH of the buffers used to study the effect of pH and redox potential on the production activity with respectively citric acid and 1 N NaOH.

5.3.3. Manometric assay

The manometric assay was used to study the effect of pH on the hydrogen production activity of the enzyme with methyl viologen semiquinone (0.3 mM) and flavodoxin hydroquinone (50 μM) as electron carriers. When methyl viologen semiquinone was used as electron carrier Warburg manometers contained in the main compartment of 6 ml: 1.8 mmol phosphate buffer pH 8-5.5, 1.8-40.1 μmol methyl viologen, 300 μmol $Na_2S_2O_4$, 67 μmol sodium(bi)sulphite and 3 mg bovine serum albumin. Sodium dithionite was dissolved anaerobically in 300 mM phosphate buffer of the same pH as the buffer used in the assay and was added to the main compartment of the manometer by syringe while flushing the manometer with argon. The hydrogenase was added under similar anaerobic conditions to the side arm. The reaction was started by tipping the enzyme from the side arm into the main compartment. Using flavodoxin hydroquinone as electron carrier Warburg manometers contained in the main compartment of 6 ml: 1.35 mmol phosphate buffer pH 8-5.5, 0.3-0.448 μmol flavodoxin, 300 μmol $Na_2S_2O_4$ and 67 μmol (bi)sulphite; dithionite was prepared as described above. The reasons for using 6 ml instead of 2 ml, as used routinely in the Warburg manometer have been described [20].

5.3.4. Spectrophotometric assay

The spectrophotometric assay was used to study the effects of pH and redox potential on the hydrogen production activity at fixed reduced electron donor concentrations. Using methyl viologen semiquinone (0.3 mM) as electron donor the activities were calculated from the decrease in absorbance at 604 nm [20] ($\varepsilon_{604\ nm}$ = 13.6 $mM^{-1}cm^{-1}$ [21]). Anaerobic cuvettes (0.4 cm light path) were stoppered with a Suba-seal rubber cap and contained in a final volume of 1.8 ml: 0.54 mmol phosphate buffer pH 8-5, 1.8-18 μmol $Na_2S_2O_4$, 0.9-200 μmol sodium (bi)sulphite, 0.545-54.5 μmol methyl viologen and 0.9 mg bovine serum albumin. It was observed that, at high methyl viologen and (bi)sulphite concentrations before addition of dithionite, a green colour developed which probably is a product of a reaction between methyl viologen and (bi)sulphite. However, the concentration of this product was apparently sufficiently low that, after addition of dithionite, it did not markedly affect the extent of reduction of methyl viologen. The nature of this product was not investigated. As has been shown by Yu and Wolin [24] at low pH methyl viologen semiquinone is only stable when the ratio of oxidized methyl viologen/semiquinone is high. This condition was met as can be derived from Table 1 and

Fig. 2A (see Results).

When flavodoxin hydroquinone (50 μM) was used as electron donor the activities were calculated from the increase in absorbance of the semiquinone at 580 nm (ε_{580} = 4.5 $mM^{-1}cm^{-1}$ [23]). Anaerobic cuvettes (1 cm light path) contained in a total volume of 1.0 ml: 0.225 mmol phosphate buffer pH 8-5, 2-6 μmol $Na_2S_2O_4$, 0.8-170 μmol sodium(bi)sulphite, 50-680 nmol flavodoxin. The cuvettes plus contents, but minus dithionite and hydrogenase, were made anaerobic by five cycles of evacuation and filling with argon gas; after the final cycle the cuvettes were filled with argon, and equilibrated at 30°C. Dithionite was then added and the absorption at 604 or 580 nm was read, when respectively methyl viologen semiquinone or reduced flavodoxin hydroquinone were used as electron carriers, followed by addition of hydrogenase. At each pH value and for both electron carriers tested, it was established that the activity was linear with the enzyme concentration, indicating that the rate of reduction of the electron carriers by dithionite was faster than the rate of oxidation of the electron carriers by hydrogenase, the latter being the rate limiting step (for detail see [20]).

The spectrophotometric assay was also used to study the effect of the concentration of flavodoxin hydroquinone at a redox potential of -381 mV ± 3 mV at pH 5.5 on the hydrogenase activity. In a total volume of 1.0 ml the anaerobic cuvettes (1 cm light path) contained 0.225 mmol phosphate buffer pH 5.5, 10 μmol $Na_2S_2O_4$, 4.05 μmol sodium(bi)sulphite and 12.9-771 nmol flavodoxin, the concentration of vlavodoxin hydroquinone ranged from 10-575 μM.

As has been shown in the accompanying paper [20], the best values for the activity are obtained at the initial stage of the activity progress curve; in other words when the increase in the concentration of the oxidized form of the electron carrier is small (ΔA_{580} = 0.002). To increase the sensitivity of the measurement the split beam mode of the Aminco DW 2a spectrophotometer was used.

5.3.5. Effect of pH on the hydrogen oxidation activity

The pH-dependence of the hydrogen oxidation activity was assayed by spectrophotometric measurement of the rate of reduction of methyl (1 mM) and benzyl viologen (1 mM) as described previously [17] at 604 and 540 nm respectively, using an absorbance coefficient of 13.05 $mM^{-1}cm^{-1}$ for reduced benzyl viologen [17]. The buffers used were 150 mM phosphate buffer pH 6-8, 150 mM Tris-HCl buffer pH 8-9 and 150 mM glycine-NaOH buffer pH 9-12.

In order to scavenge residual oxygen glucose and glucose oxidase were added

below pH 9, and above this pH 2-mercaptoethanol and vitamin B_{12}. Hydrogen oxidation activity was determined as described before [17]. It was observed that, above pH~9, after evacuation and filling the cuvettes with hydrogen gas and before addition of 2-mercaptoethanol and vitamin B_{12} derivative, thus in the absence of hydrogenase, benzyl viologen semiquinone was formed spontaneously. Hydrogen oxidation activities were corrected for this endogenous activity. Control experiments showed that this spontaneous formation of benzyl viologen semiquinone did not depend on the nature of the buffer, but only on pH; it precipitated slowly from solution as a deep purple precipitate.

5.4. RESULTS

5.4.1. Effects of temperature on the midpoint potential and pK' for flavodoxin hydroquinone

As has been shown by Mayhew [25] at low pH flavodoxin hydroquinone becomes protonated according to

$$Fl{H_2}^- + H^+ \rightleftarrows FlH_3 \qquad (2)$$

with a value for the ionization constant at 25°C of $pK' = 5.8$. However, since the determination of the hydrogen production activity with flavodoxin hydroquinone was performed at 30°C and at pH values around pH 5.8 the effects of temperature on the midpoint potential and ionization constant of flavodoxin hydroquinone were determined.

According to the corrected value, the effective midpoint potential of the dithionite/(bi)sulphite redox couple changes by $-2.2\ mV.K^{-1}$ [26]. With this value the effects of temperature on E_B (the apparent potential at pH = 0 for the unprotonated species), E_1 and pK' were determined where, according to Mayhew [25]:

$$E_1 = E_B + \frac{RT}{F} \ln(1 + \alpha\ [H^+]) \qquad (3)$$

and

$$K' = \frac{1}{\alpha} = \frac{[H^+]\ [Fl{H_2}^-]}{[FlH_3]} \qquad (4)$$

The effects of temperature on equilibrium mixtures that contained flavodoxin and excess dithionite and (bi)sulphite were examined at pH 7.5, 6.2 and 5.85 (Fig. 1A). From the temperature curves at pH 6.2 and 5.85 the values for pK' and E_B

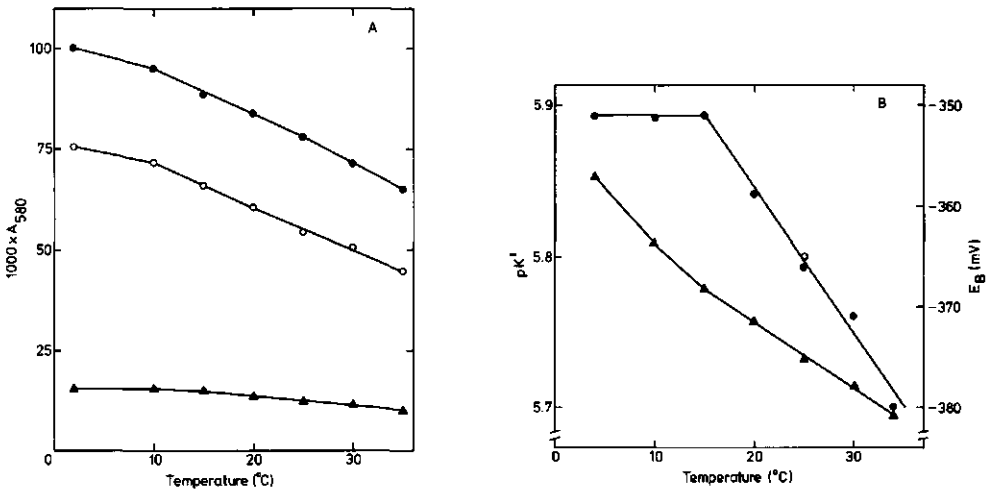

Figure 1. *Effects of temperature on the extent of reduction of* M.elsdenii *flavodoxin, on* pK' *and on* E_B, *at several pH values in the presence of excess dithionite and (bi)sulphite.*
(A) Anaerobic cuvettes contained in 3.5 ml 0.3 M phosphate buffer at the pH indicated (▲) pH 7.5, 100 µM flavodoxin and 17.7 mM (bi)sulphite; (o) pH 6.2, 40 µM flavodoxin, 5.0 mM (bi)sulphite; (●) pH 5.85, 40 µM flavodoxin, 5.0 mM (bi)sulphite. The concentration of dithionite was in all cases 1.25 mM. The temperature of the cuvette was decreased to 4°C, then increased stepwise to 35°C. The absorbance at 580 nm was read after each change in temperature. (B) The values for E_B and K' for the effective potential of dithionite-(bi)sulphite mixtures [26]: (●) pK'; (0) pK' as determined by Mayhew [25]; (▲) E_B.

could be calculated at each temperature (Fig. 1B). These values are in good agreement with the values calculated at pH 7.5, where only about 2% of the flavodoxin hydroquinone is in its protonated form. From these data it can be concluded that the change in midpoint potential of the protonated species is greater than the change in midpoint potential of the unprotonated species per K. Above 15°C $\Delta E_B.K^{-1}$ is -0.6 mV and $\Delta pK'.K^{-1}$ is -9.7×10^{-3}. From these data it can be calculated that at 30°C the value for E_B = -378 mV, with an ionization constant pK' = 5.75. These values are used throughout this paper.

5.4.2. Effect of pH on the manometrically determined hydrogen production activity

The effect of pH on the hydrogen production activity with methyl viologen semiquinone (0.3 mM) and flavodoxin hydroquinone (50 µM) is shown in Table 1 and Fig. 8.

With respect to the activity measurements determined manometrically as well

Table 1 *Effect of pH on the hydrogen production activity of* M.elsdenii *hydrogenase with methyl viologen semiquinone (0.3 mM) and flavodoxin hydroquinone (50 µM) at varying redox potential.*
Hydrogen production activity measurements were performed as described in Material and Methods. It should be noted that the activities were performed in phosphate rather than in Tris-HCl buffer [17]. For example, the activities determined in Tris-HCl buffer pH 8 with 0.3 mM methyl viologen semiquinone and 50 µM flavodoxin hydroquinone are 239 and 201 $\mu mol.min^{-1}.mg^{-1}$ respectively. All values are the average of two duplicate measurements.

Electron donor							
methyl viologen semiquinone				flavodoxin hydroquinone			
pH	E_h mV	$[MV^{2+}]$ mM	Activity $\mu mol.min^{-1}.mg^{-1}$.	pH	E_h mM	[F1] µM	Activity $\mu mol.min^{-1}.mg^{-1}$.
8.00	-592	0.300	347	8.00	-592	50	48.8
7.50	-532	0.312	590	7.56	-540	50.1	79.2
7.30	-513	0.326	731	7.40	-523	50.2	95.6
6.84	-470	0.433	1235	7.02	-486	50.8	248
6.53	-444	0.660	1990	6.75	-462	51.8	365
5.95	-404	1.96	3480	6.25	-417	58.5	726
5.50	-375	5.35	4200	5.85	-390	67.4	826

as spectrophotometrically it should be emphasized that these were performed in phosphate instead of Tris-HCl buffer, the latter buffer was used routinely in earlier studies [17,26]; both buffers affect the production activity [17]. For example, if 300 mM phosphate buffer pH 8 is used (this paper) instead of 75 mM Tris-HCl buffer pH 8, the manometrically determined hydrogen production activity with 0.3 mM methyl viologen semiquinone increases by 45%. This was previously also observed with 1 mM methyl viologen semiquinone [17]. However, the hydrogen production activity with 50 µM flavodoxin hydroquinone decreases by 75% using 225 mM phosphate buffer pH 8 (this paper) instead of 75 mM Tris-HCl buffer pH 8. The inhibition of the production activity with flavodoxin hydroquinone in phosphate buffer compared to Tris-HCl buffer is probably caused by the difference in ionic strength between these buffers, since the hydrogen production activity with flavodoxin as determined in Tris-HCl pH 8 is also inhibited by sodium chloride [17].

Since, at a given pH the effect of the redox potential on the hydrogen production activity was studied by changing the ratio dithionite/(bi)sulphite (see below), the effect of (bi)sulphite on the hydrogen production activity at pH 8 was also determined manometrically, because salts influence the hydrogenase activity [17,26]. It appears that in 300 mM phosphate buffer pH 8 addition of 100 mM (bi)sulphite stimulates the hydrogen production activity with 0.3 mM methyl viologen semiquinone by 8%; in 225 mM phosphate buffer pH 8 addition of 100 mM (bi)-sulphite inhibits the hydrogen production activity with 50 μM flavodoxin hydroquinone by 10%.

5.4.3. Effects of pH and redox potential on the spectrophotometrically determined hydrogen production activity

The effects of pH and redox potential on the hydrogen production activity with methyl viologen semiquinone (0.3 mM) and flavodoxin hydroquinone (50 μM) are shown in Fig. 2A and 2B respectively. From both figures it appears that, firstly, the activity increases at decreasing pH, secondly, at a given pH the activity is independent of the redox potential up to a certain value, after which the activity declines, thirdly, at decreasing pH values, this redox potential-independent part of the hydrogen production activity shifts to more positive potentials, fourthly, the redox potential at which hydrogenase does not produce hydrogen anymore increases with decreasing pH and fifthly, in the range of redox potentials where the hydrogen production activity is constant, at less negative redox potentials, the concentration of the reduced electron carrier is constant while the concentration of the oxidized form of the carrier increases. This latter observation suggests that, firstly, the oxidized forms of the electron carriers used do not inhibit the hydrogenase and secondly, that flavodoxin semiquinone cannot serve as electron donor for hydrogenase to produce hydrogen. Further it is noticeable that at a given pH the redox potential at which half of the maximal activity at that pH is observed is at the potential of the hydrogen electrode at that pH (see Fig. 3). (Bi)sulphite affects the hydrogen production activity with methyl viologen semiquinone and flavodoxin hydroquinone. However, since this effect is rather small (see above), and because at pH values below pH 7 the (bi)sulphite concentration did not exceed 100 mM, the effect of (bi)sulphite on the patterns as observed in Fig. 2A, 2B and Fig. 6 may be assumed to be negligible.

The pH-dependence of the redox potentials at which hydrogen is no longer

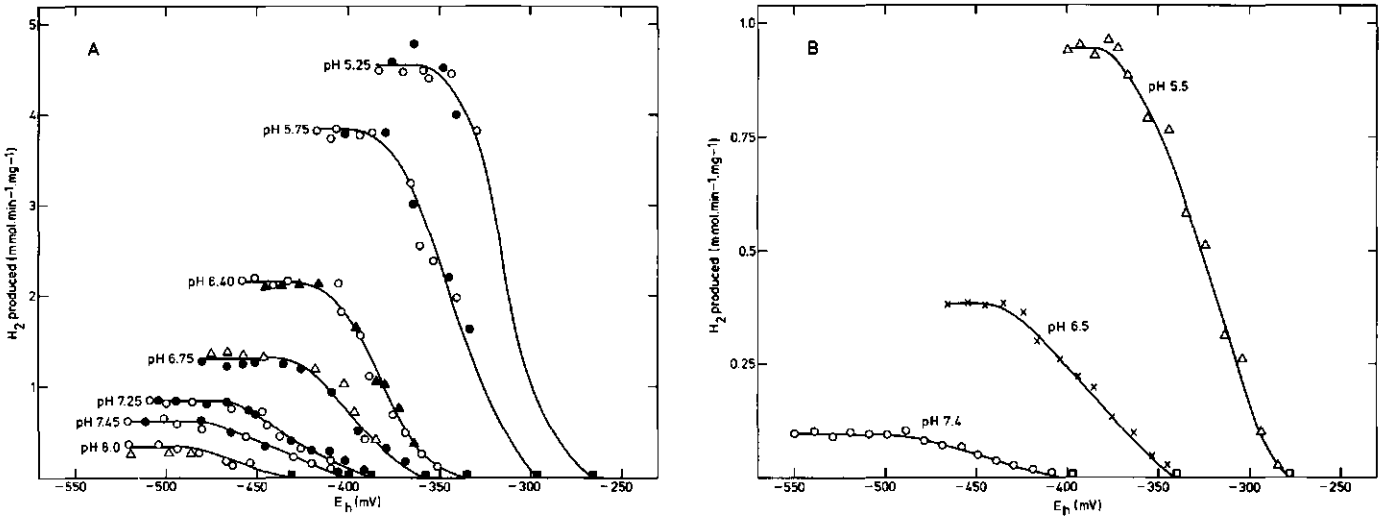

Figure 2. *Effects of pH and redox potential on the hydrogen production activity with methyl viologen semiquinone and flavodoxin hydroquinone.*
(A) Activities with methyl viologen (0.3 mM). Activities were determined spectrophotometrically, from the decrease in absorbance at 604 nm and calculated according to ref. [20]. Conditions are as described in Material and Methods. The dithionite concentrations used were: (●) 1 mM; (○) 2 mM; (X) 3 mM; (Δ) 5 mM and (▲) 10 mM; (■) end potentials. All values were the average of duplicate measurements. (B) Activities with flavodoxin hydroquinone (50 μM). Activities were determined spectrophotometrically from the increase in absorbance at 580 nm and calculated according to ref. [20]. Conditions are as described in Material and Methods. Symbols are as for Fig. 2A; (□) end potentials. All values are the average of duplicate measurements.

produced, the end potentials, were determined independently with both electron donors. The effect of pH on the end potential is shown in Fig. 3. From this figure it can be clearly seen that the end potentials become more positive at

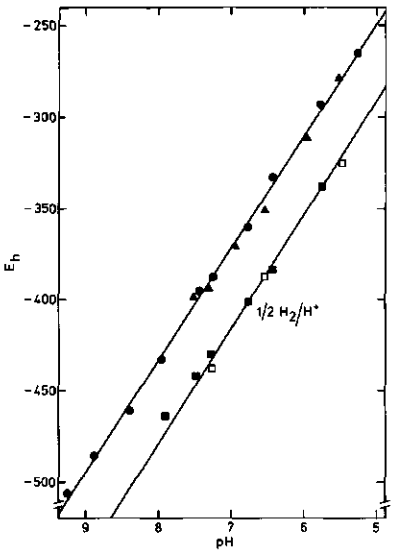

Figure 3. *Effects of pH on the end potentials.*
Anaerobic cuvettes contained in a final volume of 1.8 ml: 300 mM phosphate buffer pH 8-5, 200 mM Tris-HCl buffer pH 8-9, either 0.3-20 mM methyl viologen or 50-58 µM flavodoxin, 2-20 mM (bi)sulphite and 0.5 mM dithionite. Next hydrogenase was added and the cuvettes were stored in an anaerobic chamber filled with argon at 30°C during 24 hrs. After this period the absorbance at 604 (methyl viologen semiquinone) or 580 nm (flavodoxin semiquinone) was measured and the end potentials calculated. Storage for another 24 hrs did not change the degree of oxidation indicating that no more hydrogen was produced. (●) end potentials observed with methyl viologen semiquinone as electron donor; (▲) end potentials observed with flavodoxin hydroquinone as electron donor; (■) potential at which half of the redox potential independent activity is observed with methyl viologen semiquinone as electron donor at the corresponding pH: the 'apparent midpoint potential' (derived from Fig. 2A); (□) potential at which half of the redox potential independent activity is observed with flavodoxin hydroquinone as electron carrier at the corresponding pH (derived from Fig. 2B).

decreasing pH. The slope of Δ (end potential)/ΔpH is -61 mV and thus parallels the hydrogen electrode; the end potentials are, on average, 58 mV more positive than the hydrogen electrode at the corresponding pH. Further, it is noticeable that the redox potentials at which the hydrogen production activity ceases to be

redox potential-independent (Fig. 2) are, at the pH values tested, about 45 mV more negative than the hydrogen electrode at the corresponding pH values.

The effects of pH and redox potential on the hydrogenase activity are observed with the artificial electron donor methyl viologen semiquinone and with the natural electron donor flavodoxin hydroquinone. This indicates that the effects described do not depend on the nature of the electron donor, but rather represent effects of pH and redox potential on the hydrogenase activity itself. For *Clostridium pasteurianum* hydrogenase Chen [27] also observed a dependence of the end potentials on the pH. From his data it can be derived that at pH 8 and pH 7.2 these end potentials are about 60 mV less negative than the potential of the hydrogen electrode at the particular pH values, all in agreement with the data for *M.elsdenii* hydrogenase. However, at pH 6 a strong deviation from this relation can be calculated.

5.4.4. Kinetics with flavodoxin at pH 5.5

In contrast to our expectation a double reciprocal plot of the concentration of flavodoxin hydroquinone versus the activity is non-linear (Fig. 4). At low pH a linear double reciprocal plot was expected for the activity versus the electron donor concentration, since both models proposed for hydrogen production [17] predict that at low pH the pathway will become compulsory with respect to the order of addition of protons and electrons. As can be derived from Table 2, the activity curve could be fitted equally well for Eqn (6) as for Eqn (7) of ref. [17]. Compared with the production activity at pH 8 the value for V increases about fourfold while the $[S]_{0.5}$ value decreased slightly from 150 μM to about 100 μM. However, this fourfold increase in V is probably an underestimation since the hydrogen production activity determinations were performed in phosphate buffer which inhibits the production activity with flavodoxin compared with Tris-HCl buffer, as described above. From Fig. 1B the ratio of the protonated (FlH_3) and unprotonated (FlH_2^-) forms of flavodoxin hydroquinone can be calculated. Assuming that the activity of hydrogenase towards these two species is independent and additive, and both activities fulfill Eqn(7) of ref. [17] the activity of the enzyme towards [FlH_3] and [FlH_2^-] could be simulated (Table 2). The $[S]_{0.5}$ value for both substrates is about 50 μM, while the value for V for FlH_3 is twice that for FlH_2^-.

Table 2. *Results of fitting the kinetic data at pH 5.5 for flavodoxin according to Eqns (6) and (7) of ref.* [17].
Activity measurements were performed as described in Material and Methods. x_1-x_6 are rate constants according to Eqn (6) or Eqn (7) of ref. [17]. The concentration range given is that of *M.elsdenii* flavodoxin hydroquinone used to test the hydrogenase activity. The number of experiments performed at different donor concentration is 20.

Parameter	Unit	Value for flavodoxin hydroquinone		FlH_2^-	FlH_3
x_1	$mM^3 min^{-1}$	0.057	--		
x_2	$mM^2 min^{-1}$	6.62	0.0431	0.0036	0.0194
x_3	$mM\ min^{-1}$	3.68	4.14	1.41	2.63
x_4	mM^3	0.011	--		
x_5	mM^2	0.105	0.0077	0.0026	0.0020
x_6	mM	1.56	0.0537	0.052	0.0216
$[S]_{0.5}$	mM	0.098	0.106	0.052	0.055
V		3.89	4.14	1.41	2.63
Standard error estimate %		0.2%	0.3%	0.4%	0.4%
Substrate inhibition		+	-	-	-
Concentration range: mM			0.011 - 0.575		

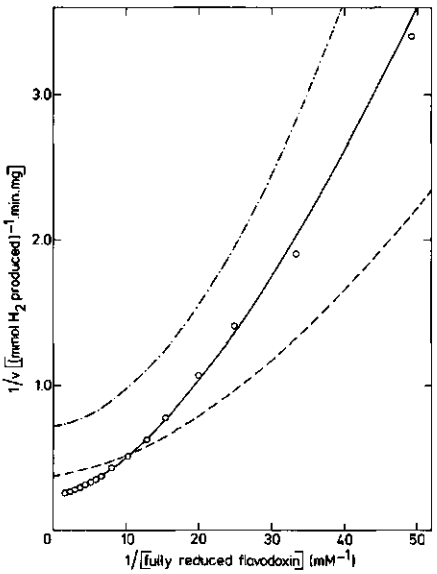

Figure 4. *Double reciprocal plot of the hydrogen production activity,* v, *on the concentration of* M.elsdenii *flavodoxin hydroquinone, at pH 5.5.*
Activities were determined spectrophotometrically from the increase in absorbance at 580 nm and calculated as given in ref. [20]. Conditions are as described in Material and Methods. (0) observed activity with flavodoxin (average of duplicate measurements); (——) calculated activity with flavodoxin according to Eqns (6) or (7) from ref. [17]; (---) calculated activity with FlH_3; (-·-·-) calculated activity with FlH_2^-.

5.4.5. pH-dependence of the hydrogen oxidation activity.

The pH-dependence of the hydrogen oxidation activity with methyl and benzyl viologen is shown in Fig. 5. For both electron acceptors the pH optimum is at pH 9.7. At this pH a double reciprocal plot of the kinetic data for benzyl viologen is linear; V is 15.2 $mmol.min^{-1}.mg^{-1}$, with a Km value of 5.0 mM. It was observed, that the ratio of the hydrogen oxidation activity with benzyl and methyl viologen as substrate is constant (5.15 ± 6%) throughout the pH range tested. The molar salt stimulation coefficients [26] with NaCl at pH 9.7 are 1500 and 7000 M $salt^{-1}.mg^{-1}$ for respectively methyl and benzyl viologen as electron acceptor. These increases in molar salt stimulation coefficients are in proportion with the increases in specific activity due to an increased pH. (pH 8 compared to pH 9.7).

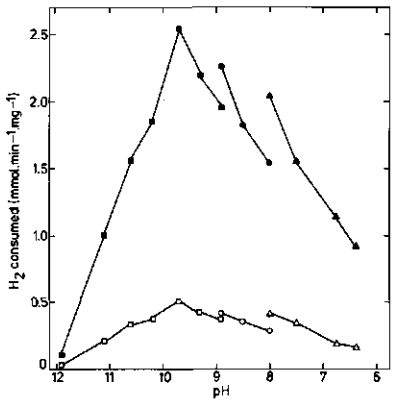

Figure 5. *Effect of pH and buffer on the hydrogen oxidation activity.* Open symbols, 1 mM methyl viologen as electron acceptor; closed symbols, 1 mM benzyl viologen as electron acceptor. (Δ) 150 mM phosphate pH 6-8; (0) 150 mM Tris-HCl pH 8-9; (□) 150 mM glycine-NaOH pH 9-12.

5.5. DISCUSSION

As is shown in Table 1, Figs. 2 and 3, the hydrogen production activity depends on the pH, on the redox potential, and on the nature of the electron donor. Since it is reasonable to assume that the ferredoxin-type clusters within the enzyme [17,26] serve as electron accepting sites, the only explanation for the dependence on pH and redox potential of the hydrogenase is that the midpoint potentials of these clusters are to a certain extent pH-dependent. For example if it is assumed that the midpoint potentials of the ferredoxin-type clusters within the enzyme, like nearly all other electron transferring ferredoxins are pH-independent [28], and transfer one electron per cluster at an average midpoint potential of -400 mV, it can be calculated that at -350 mV only 13% of the clusters are in the reduced form; nevertheless, at pH 5.25 at this potential nearly maximal activity is observed (Fig. 2A). Furthermore, at pH 8 at -430 mV 76% of the clusters are in the reduced form, but no activity is observed (Fig. 2A). These results suggest that the midpoint potential(s) of a (the) ferredoxin-type cluster(s) is (are) pH-dependent. However, from the present data it cannot be concluded that a proton is obligatorily involved in the oxidation-reduction mechanism of a (the) cluster(s), although this possibility cannot be excluded. For example, Prince and Dutton [29] showed that the midpoint potential of the Rieske iron-sulphur centre is pH-independent from pH 6-8, but becomes pH-dependent above pH 8. Recently Ingledew and Ohnishi [30] showed that the midpoint potentials of centres

N-1a and N-2 of NADH dehydrogenase are pH-dependent.

Our data indicate that at each pH for both substrates tested (methyl viologen semiquinone and flavodoxin hydroquinone), the difference in potential between the end of the redox potential independent part of the activity and the end potential is about 100 mV (Fig. 2A,B). If, at a given pH, the activities are considered to express the degree of reduction of the enzyme, with the redox potential-independent part of the activity representing full (100%) reduction and the end potentials representing complete oxidation (0% reduction), a n=2 type of redox titration curve is obtained (Fig. 6). These n=2 type redox titration curves are observed at the pH range tested and for both electron donors (Fig. 6) and are thus a property of the enzyme.

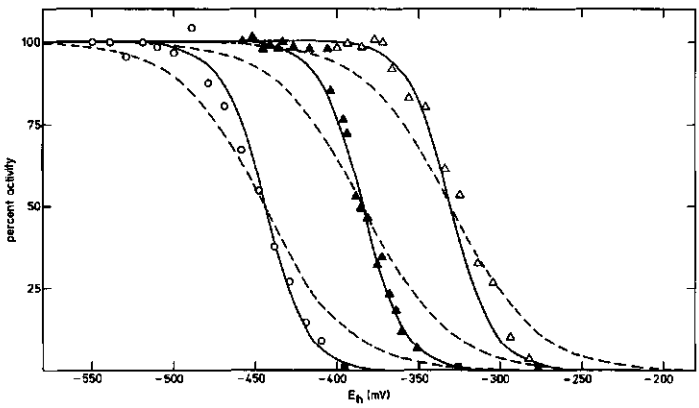

Figure 6. *Redox dependent hydrogen production activities as related to redox titrations.*
It was assumed that in Fig. 2 the activities of the redox independent part represent full reduction, and the end potentials complete oxidation of the enzyme. Activities are expressed as percentage of the activities obtained at the redox independent part. (o) observed activities with flavodoxin hydroquinone at pH 7.4 (Fig. 2B); (▲) observed activities with methyl viologen semiquinone at pH 6.4 (Fig. 2A); (Δ) observed activities with flavodoxin hydroquinone at pH 5.5; in relation to (——) n=2 type redox titration curve; (---) n=1 type redox titration curve. The 'apparent midpoint potential' for the redox titrations curves are at -0.444, -0.384 and -0.330 V.

The mechanistic model of Fig. 7, which is an adaptation of our Model I (cf. [17]), explains the n=2 type redox behaviour together with the observed change of the apparent midpoint potential of -60 mV/pH unit, and has the same mathematical formulation of the rate equation. The main difference between the model as proposed in Fig. 7 and the earlier proposed models [17] is that the previous models suggest that a proton is in the immediate vicinity of its electron-

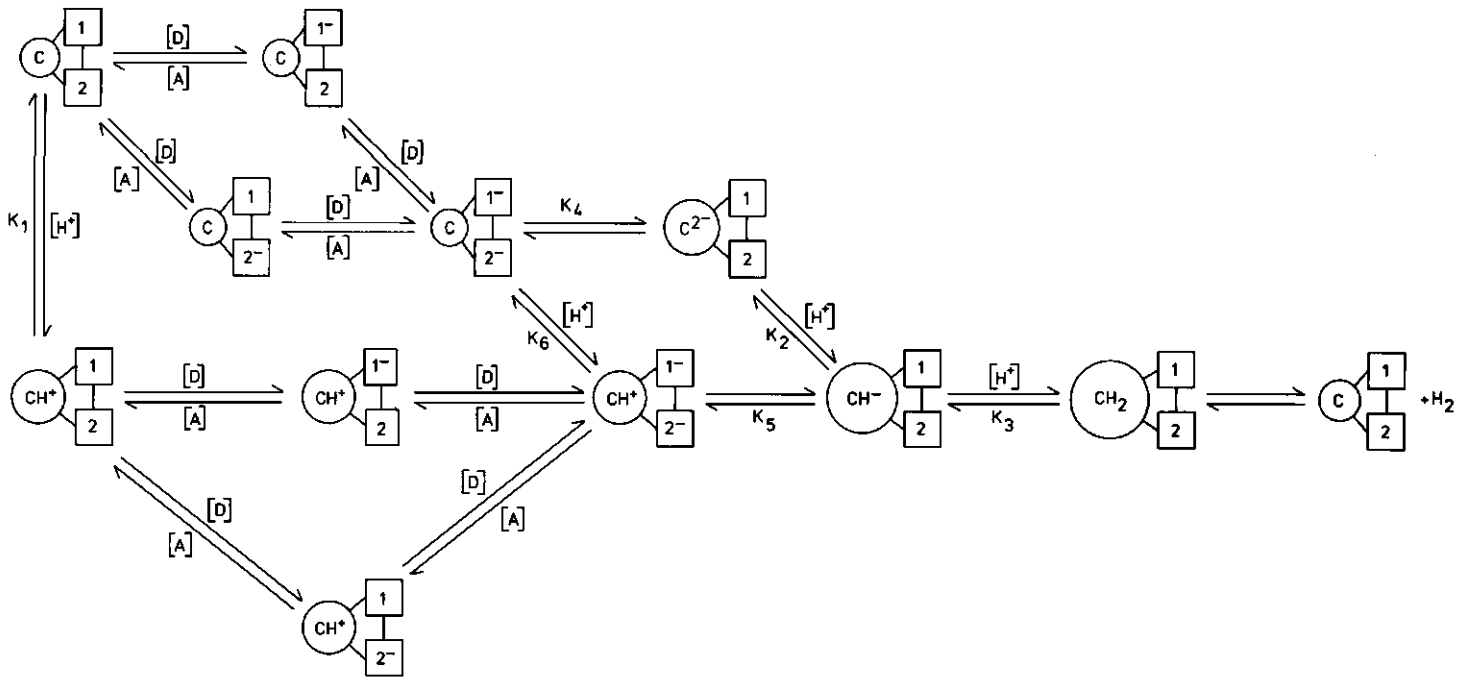

Figure 7. *Adapted model for* M.elsdenii *hydrogenase activity.*
[D] reduced electron donor concentration; [A] oxidized electron donor concentration; 1, 2, oxidized ferredoxin-type clusters; 1^-, 2^-, reduced ferredoxin-type clusters; C, catalytic centre; CH^+, C^{2-}, CH^-, CH_2, respectively proton at the level of the catalytic centre, two electrons at the level of the catalytic centre, catalytic centre-hydride complex, catalytic centre-hydrogen complex. K_1, K_2, K_3, K_6 equilibrium constants according to Eqns (12,17,18,13) respectively. K_4, K_5 constants describing inter-enzymic redox processes as described in the text.

accepting site(s), suggesting the formation of native hydrogen at the level of the ferredoxin-type clusters. This suggestion is not in agreement with a n=2 type redox titration curve, if it is supposed that the two ferredoxin-type clusters are each transferring one electron. The kinetic model of Fig. 7 proposes that both ferredoxin-type clusters need to be reduced in two one-electron reduction steps after which two electrons are transferred to the catalytic centre, which accepts two electrons at the same redox site, and hydrogen production occurs. This model further supposes protonation of the catalytic centre of the oxidized species, all in accordance with the earlier proposed models. Protonation equilibria might also exist between two species of the enzyme with the same state of reduction, but for reasons of simplicity these steps have not been included in Fig. 7. However, each protonation step extends the contribution of the terms of [D] in the numerator and denominator with one power.

The explanation for the n=2 type of redox behaviour of the enzyme must take into account that, firstly, all experiments were performed under initial steady-state conditions, with all enzyme species in equilibrium, and no hydrogen present. Secondly, the electron donor concentration D was also constant in all experiments so that (Σk_d [D]) is constant, where k_d is the rate constant associated with D. Thirdly, the redox potential is increased by increasing the concentration of A, so that the value of Σk_a [A], where k_a is the rate constant associated with A, increases at increasing redox potential. Fourthly, the general rate equation for the initial hydrogen production for the kinetic model of Fig. 7 is that of Eqn (6) of ref. [17]. However, to determine the effects of pH and E_h on the hydrogen production activity, both D and A are present. In the presence of A the general rate equation for the hydrogen production activity is a very complex function of A and D:

$$\frac{v}{[E]_t} = \frac{[D]^2(x_1 + x_7[A] + x_8[A]^2) + [D]^3(x_2 + x_9[A]) + x_3[D]^4}{\{[A](x_{10} + x_{11}[A] + x_{12}[A]^2 + x_{13}[A]^3) + [D](x_4 + x_{14}[A] + x_{15}[A]^2 + x_{16}[A]^3) + [D]^2(x_5 + x_{17}[A] + x_{18}[A]^2) + [D]^3(x_6 + x_{19}[A]) + x_7[D]^4\}} \quad (7)$$

In the absence of A Eqn (7) simplifies to Eqn (6) of ref. [17]. The observation that for the redox-independent part of the hydrogen production activity, for both substrates, and for all pH values tested (Fig. 2) no detectable decrease in activity is observed can be explained if in Eqn (7) the contribution of the terms

$x_i[A]^n$ (x_i, a set of rate constants associated with A; n, integer from 1-4) is a negligible contribution to the terms $x_j\ [D]^n$ (x_j, a set of rate constants associated with D; n, integer from 1-4). When the hydrogen production activity becomes redox potential-dependent, the contribution of the terms $x_i\ [A]^n$ apparently becomes of such an importance that the activity v decreases and even stops at the end potential. At decreasing pH the values of x_i and x_j apparently change in such a way, that the concentration of A necessary to inhibit the hydrogen production activity has to be larger.

For the following part of the discussion it should be emphasized that, to explain the effects of pH and E_h on the hydrogen production activity on the basis of the model of Fig. 7, it is assumed that, firstly, the ratio of D/A not only determines the hydrogen production activity according to Eqn (7) but also the redox potential E_h. Secondly, that the ferredoxin-type clusters within the enzyme each serve as electron-transferring sites and obey the redox potential of the D/A redox couple. Thirdly, that the redox potential of the 'two-electron reduced catalytic centre'/'oxidized catalytic centre' is that of the reduced/oxidized ferredoxin-type clusters, and therefore equals the redox potential of the D/A redox couple. In other words this suggests that the hydrogenase contains two distinguishable, separate types of redox centres, which are in redox equilibrium with each other.

As has been clearly demonstrated at a given pH the change in activity of the enzyme with respect to the increase in redox potential is independent of the nature of the electron donors (methyl viologen semiquinone, or flavodoxin hydroquinone) and thus a property of the enzyme.
This points to the fact that, in view of the initial steady-state conditions applied, all intermediates of the enzyme are in equilibrium and that both types of redox centres within the enzyme are in redox equilibrium with the D/A redox couple. It can thus be said that:

$$E_h = E_{m1} + \frac{RT}{F}\ln\frac{[A]}{[D]} = E_{m2} + \frac{RT}{nF}\ln\frac{[\text{E-Fd}_{ox}]}{[\text{E-Fd}_{red}]}$$

$$= E_o + \frac{RT}{2F}\ln\frac{\Sigma[CH^+][H^+]}{[CH_2]} \qquad (8)$$

E_o, $E_{m1,2}$, are the midpoint potentials of the particular redox couples. E-Fd_{ox} and E-Fd_{red} are the oxidized and reduced forms of the ferredoxin-type clusters (for example, the C^{2-} form represents an oxidized ferredoxin-type enzyme species in which the two-electrons are located at the level of the catalytic centre);

it is not known whether protonation of the catalytic centre affects the midpoint potential(s) of a (the) ferredoxin-type cluster(s). $\Sigma[CH^+]$ are those enzyme species where the oxidized catalytic centre is protonated, thus also including those enzyme species where a (the) ferredoxin-type cluster(s) is (are) reduced; CH_2 is the enzyme hydrogen complex. With respect to n in Eqn (8) this value is 1 when the ferredoxin-type clusters show a redox potential-independent behaviour and when these clusters show a redox dependent-behaviour n is 1 to 2.

Clark [31] has developed a systematic formulation of redox equations that involves the coupling of electron transfer with proton transfer. A similar treatment was used here, as outlined below, to obtain a redox equation which describes the redox titration curves (Fig. 6) on the basis of the kinetic model of Fig. 7. Since the redox titration curves of Fig. 6 are based on activity determinations, which represent a property of the catalytic centre, together with the assumed similarity in redox potentials as described in Eqn (8), the following theoretical approach to explain the effects of pH and E_h on the activity only considers the catalytic centre. It is assumed that at pH = 0 the predominant oxidized and reduced enzyme species are CH^+ and CH_2, thus the protonated forms of the oxidized and two-electron reduced catalytic centre and relations will be orientated by the process:

$$CH^+ + H^+ + 2e \rightleftarrows CH_2 \qquad (9)$$

According to the notation of Clark [31] the primary redox equation derived from Eqn (9) is (at 30°C):

$$E_h = E + 0.03 \log \frac{[CH^+][H^+]}{[CH_2]} \qquad (10)$$

The total concentration of oxidized enzyme, at the level of the catalytic centre, S_o, is (see Fig. 7):

$$S_o = [CH^+] + [C] + [C_{\alpha 1}H^+] + [C_{\alpha 1}] + [C_{\alpha 2}H^+] + [C_{\alpha 2}] + [C_{\alpha 1,2}H^+] + [C_{\alpha 1,2}] \qquad (11)$$

in which $[C_{\alpha 1,2}]$ represents the enzyme species containing two electrons at the level of the ferredoxin-type clusters, $[C_{\alpha 1}]$ and $[C_{\alpha 2}]$ the species with cluster 1 reduced and cluster 2 oxidized, respectively cluster 1 oxidized and cluster 2 reduced.

For the oxidized catalytic centre two protonation equilibria can be described (see Fig. 7):

$$K_1 = [C][H^+]/[CH^+] \qquad (12)$$

$$K_6 = [C_{\alpha 1,2}][H^+]/[C_{\alpha 1,2}H^+] \quad (13)$$

Since the hydrogen production activity determinations were performed under steady-state conditions, d[enzyme intermediate]/dt = 0, so that each enzyme species has a fixed concentration during catalysis it can be said that:

$$c_1 = [C_{\alpha 1}]/[CH^+] \quad (14a)$$
$$c_2 = [C_{\alpha 2}]/[CH^+] \quad (14b)$$
$$c_3 = [C_{\alpha 1}H^+]/[CH^+] \quad (14c)$$
$$c_4 = [C_{\alpha 2}H^+]/[CH^+] \quad (14d)$$
$$c_5 = [C_{\alpha 1,2}H^+]/[CH^+] \quad (14e)$$

Substituting Eqns (12-14) into Eqn (11) and solving it for $[CH^+]$ gives:

$$[CH^+] = S_o\left(\frac{[H^+]}{K_1 + c_5K_6 + [H^+](1 + c_1 + c_2 + c_3 + c_4 + c_5)}\right) \quad (15)$$

The total concentration of reduced enzyme, at the level of the catalytic centre, S_r, is (see Fig. 7):

$$S_r = [CH_2] + [CH^-] + [C^{2-}] \quad (16)$$

For the reduced catalytic centre two protonation equilibria can be described (see Fig. 7):

$$K_2 = [C^{2-}][H^+]/[CH^-] \quad (17)$$

$$K_3 = [CH^-][H^+]/[CH_2] \quad (18)$$

Substituting Eqns (17,18) into Eqn (16) and solving it for $[CH_2]$ gives:

$$[CH_2] = S_r\left(\frac{[H^+]^2}{K_2K_3 + K_3[H^+] + [H^+]^2}\right) \quad (19)$$

Since the redox potential, E_h, of the A/D redox couple determines the redox potential of the oxidized/reduced catalytic centre (Eqn (8)) substituting Eqns (15) and (19) into Eqn (10) gives, according to the notation of Clark [31]:

$$E_h = E_o + 0.03 \log \frac{S_o}{S_r} + 0.03 \log \left(\frac{[H^+]^2 + K_3[H^+] + K_2K_3}{K_1 + c_5K_6 + [H^+](1 + c_1 + c_2 + c_3 + c_4 + c_5)}\right) \quad (20)$$

Eqn (20) explains the n=2 type redox potential-dependence at constant pH of the symmetrical redox titration curves of Fig. 6.

Fig. 8 shows the effect of the proton concentration on the hydrogen production activity with methyl viologen semiquinone and flavodoxin hydroquinone. The inserts of Fig. 8A and 8B show two points of inflection observed at pH 6.8 and pH 5.8 for methyl viologen semiquinone, and at pH 7.0 and pH 6.0 for flavodoxin hydroquinone. These points of inflection at pH≈7 and pH≈6 could represent pK values of at least two distinct amino acids, close to the catalytic centre, necessary to deliver protons for hydrogen production. If it is assumed that for both electron donors tested, the effects of protons on the enzyme activity can be neglected, and protons only serve as substrate, double reciprocal plots of the activity versus proton concentration are non-linear. The value for h is in both cases 0.85 ± 0.3, the $[S]_{0.5}$ value for $[H^+]$ is for both electron donors tested at pH 6.4 ± 0.2. Since, as derived above, the protonation constants K_2 and K_3 are $\sim 10^{-8}$ M a pattern as observed from the inserts of Fig. 8 might also be explained other than from pK values of neighbouring amino acids. At a constant electron donor concentration and varying proton concentrations, neglecting the effects of protons on the enzyme Eqn (6) of ref. [17] is also valid for a random proton addition.

As has been shown in Table 2, FlH_3 is a better substrate than FlH_2^- though the $[S]_{0.5}$ values for both substrates are almost the same. The reason for this might be that FlH_3 carries besides an electron also a proton. As can be derived from Fig. 2B and Fig. 6 the redox potential of the kinetic measurements (-381 mV ± 3 mV) was low enough to cancel the effect of the redox potential on the activity. As has been shown previously [17] and in this paper the hydrogen production activity with flavodoxin hydroquinone is inhibited by an increase in ionic strength. It should be pointed out that the activity with methyl viologen semiquinone is stimulated by a high ionic strength [17,26] however, the $[S]_{0.5}$ values for methyl viologen semiquinone monomer and dimer decrease upon increasing ionic strength [17]. Since the kinetics of the hydrogen production activity with flavodoxin at pH 8 were determined in Tris-HCl buffer and the kinetics at pH 5.5 in phosphate buffer, respectively representing a buffer with low and high ionic strength, the decrease in $[S]_{0.5}$ value might partially be due to the difference in ionic strength of these buffers.

It is noteworthy that the enzyme preincubated under oxidizing conditions (irreverisbly oxidized enzyme), which shows an increase in activity of about 1.6 times [26], shows an identical behaviour in the hydrogen production activity on changing the pH and redox potential. At each combination of pH and redox potential the hydrogen production activity is about 1.6 times higher than that of the reduced enzyme (data not shown).

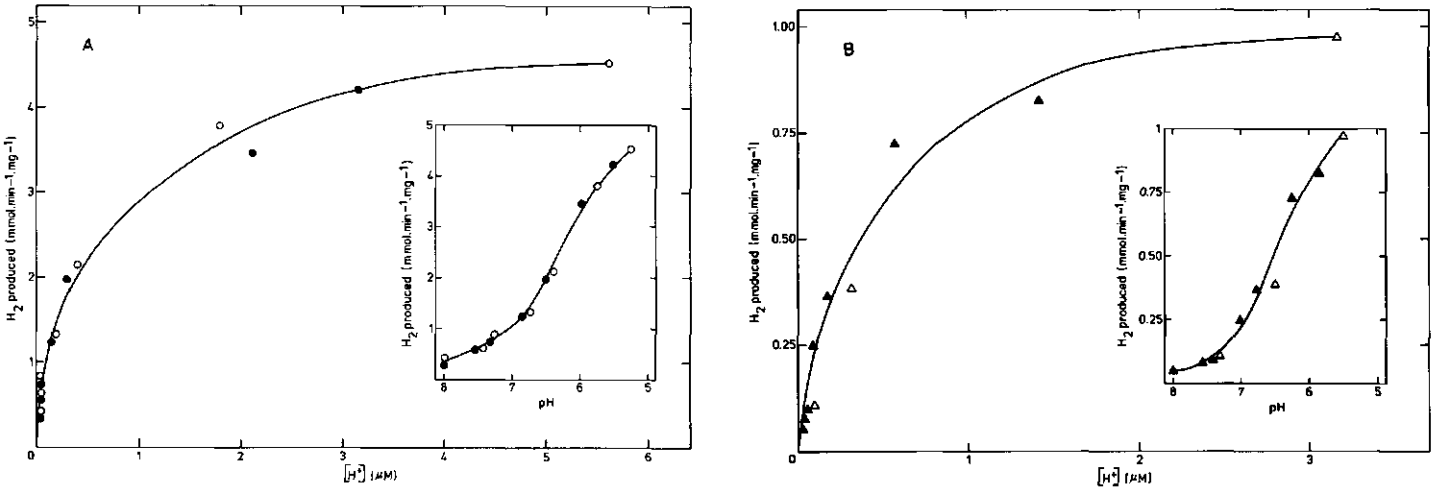

Figure 8. *Dependence of the hydrogen production activity on the proton concentration with methyl viologen semiquinone and flavodoxin hydroquinone as electron donors.*
(A) Activities with methyl viologen semiquinone (0.3 mM) as electron donor. (o) activities derived from Fig. 2A for the redox-independent part of the activities; (●) activities derived from Table 2. Insert: dependence of the hydrogen production activity on the pH; symbols are as for the figure. (B) Activities with flavodoxin hydroquinone (50 μM) as electron donor. (Δ) activities derived from Fig. 2B for the redox-independent part of the activities; (▲) activities derived from Table 2. Insert: dependence of the hydrogen production activity on the pH; symbols are as for the figure.

If it is assumed that the kinetics of the hydrogenase with methyl viologen semiquinone [17] are unaltered at low pH values a turnover number of 0.8-1.7 x 10^5 moles H_2 produced (sec.mole hydrogenase)$^{-1}$ can be calculated. The fact that the extrapolated turnover number with flavodoxin hydroquinone 0.4-1.6 x 10^4 moles H_2 produced (sec.mole hydrogenase)$^{-1}$) is an order of magnitude lower indicates the existence of a flavodoxin hydroquinone-hydrogenase enzyme-substrate complex. The turnover numbers for the hydrogen oxidation activity with benzyl and methyl viologen are respectively 1.3 x 10^4 (calculated) and 5.9 x 10^4 (estimated) moles H_2 oxidized (sec.mole hydrogenase)$^{-1}$.

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6. SUMMARY

This thesis is concerned with the purification and properties of hydrogenase from the obligate anaerobic rumen bacterium *Megasphaera elsdenii*. In chapter 1 the motives underlying this thesis, the physiological role of hydrogenase in some heterotrophs, including *Megasphaera elsdenii*, as well as a comparison of physico-chemical and kinetic properties of purified hydrogenase preparations from microorganisms, showing great variations in physiology and taxonomy, are given. The physiological role and the physico-chemical and kinetic properties of the hydrogenases from *Megasphaera elsdenii* and *Clostridium pasteurianum* show great similarities.

In chapter 2 the procedure for the anaerobic purification of hydrogenase from *Megasphaera elsdenii* is given. Two activity bands are separated on DEAE-cellulose chromatography, of which one (fraction I; see Table 1 of this chapter) represents a different hydrogenase or a complex of the enzyme in fraction II. Kinetics with the purified enzyme of the hydrogen production activity at pH 8, with the electron donors flavodoxin hydroquinone, reduced ferredoxin and methyl viologen semiquinone show non-linear double reciprocal plots of the activity versus the electron donor concentration. Two kinetic models were developed, with an identical general rate equation for the hydrogen production activity, which describe a random mechanism for the reaction of the oxidized enzyme with a proton and a reduced electron donor. These models also indicate that the hydrogenase accepts the two electrons necessary for hydrogen production in two separate, independent steps. Hydrogen oxidation with methyl and benzyl viologen shows, in contrast to hydrogen production, Michaelis Menten kinetics. In chapter 3 effectors of the hydrogenase activity, such as salts (which have hydrophylic properties) Me_2SO and ethylene glycol (which have hydrophobic properties), and oxidants (oxygen, ferricyanide, Cl_2Ind, (bi)sulphite) are described. The data show that the more chaotropic the anion of the salt the greater the increase in activity, per mole salt used. Both Me_2SO and ethylene glycol inhibit the hydrogen evolution activity, however, Me_2SO stimulates the hydrogen oxidation activity, while ethylene glycol does not affect this activity. Careful oxidation of the reduced enzyme with (bi)sulphite or Cl_2Ind irreversibly increases the activity of the en-

zyme by about 60%; in contrast, oxygen and ferricyanide inactivate the enzyme. This irriversibly oxidized enzyme, which shows identical kinetic properties to the reduced enzyme, is more resistant to the effects of oxygen, Me_2SO and storage, than the reduced enzyme. Difficulties encountered in ascribing redox potentials to the observed EPR spectra of hydrogenase at several redox states, such as the effects of temperature on the Nernst equation, on the apparent pH and on the midpoint potentials of the redox species, are described.

In chapter 4 the theoretical aspects are described to determine the hydrogen production activity spectrophotometrically in a series coupled redox reactions as function of the pH and redox potential, as well as the limitations of this method. The hydrogen production activity is determined from the decrease in concentration of the reduced electron donor, or increase in the concentration of the oxidized electron donor. The changes in concentration of the reduced or oxidized electron donor do not equal the amount of hydrogen produced but only represent a measure of the actual amount of hydrogen produced.

In chapter 5 the method as described in chapter 4 is used to study the effects of pH and redox potential on the hydrogen production activity. The hydrogen production activity is strongly pH- and redox potential-dependent; the activity patterns observed as function of pH and redox potential are independent of the nature of the electron donor used and thus represent a property of the enzyme. If at a given pH the activities are considered to express the degree of reduction of the enzyme the dependence of the activity on the redox potential represents a n=2 type redox titration curve with an 'apparent midpoint potential' which corresponds with the potential of the hydrogen electrode at that pH. To explain the effects of redox potential, proton and electron donor concentration on the hydrogenase activity Model I of chapter 2 was slightly adapted without changing its general rate equation for the hydrogen production activity. This model also explains the 'loss' of electrons of the reduced enzyme in EPR spectroscopy as well as the formation of HD together with DD during H_2-D_2O exchange experiments.

7. SAMENVATTING

Dit proefschrift beschrijft de zuivering en enige eigenschappen van het hydrogenase geïsoleerd uit de obligaat anaerobe pens-bacterie *Megasphaera elsdenii*.

In hoofdstuk 1 worden de motieven, die aan dit onderzoek ten grondslag liggen, gegeven. Eveneens wordt een vergelijking gemaakt, aan de hand van fysisch-chemische en kinetische eigenschappen, tussen gezuiverde hydrogenase preparaten, geïsoleerd uit micro-organismen, die onderling een grote variatie in fysiologie en taxonomie vertonen. Uit deze vergelijking blijkt, dat de fysiologische rol en de fysisch-chemische en kinetische eigenschappen van de hydrogenasen geïsoleerd uit *M.elsdenii* en *C.pasteurianum* grote overeenkomsten vertonen.

In hoofdstuk 2 wordt de procedure beschreven voor de anaerobe zuivering van het *M.elsdenii* hydrogenase. Het blijkt, dat bij chromatografie op een DEAE-cellulose kolom twee fracties worden gescheiden, die beide hydrogenase activiteit bevatten. Uit één fractie wordt het in dit proefschrift beschreven hydrogenase geïsoleerd. Tot nu toe is het nog niet gelukt een hydrogenase uit de andere fraktie te isoleren. Het is niet duidelijk of het nog niet gezuiverde hydrogenase een ander enzym is, of een complexe vorm van het gezuiverde enzym. Kinetiek met het gezuiverde enzym van de waterstof productie activiteit bij pH 8, met de electron-donoren flavodoxine hydroquinon, gereduceerd ferredoxine en methyl viologeen semiquinon laat zien dat het enzym niet voldoet aan Michaelis-Menten kinetiek. Om het kinetisch gedrag te verklaren werden twee modellen ontworpen, waarvan de algemene vergelijking voor de waterstof productie activiteit identiek is. Deze modellen beschrijven een mechanisme, waarbij het geoxideerde enzym willekeurig kan reageren met een proton of een gereduceerd electron-donor. Deze modellen beschrijven eveneens, dat het hydrogenase de twee electronen die nodig zijn om waterstof te kunnen vormen opneemt, in twee gescheiden, onafhankelijke stappen. In tegenstelling tot de waterstof productie activiteit vertoont de waterstof oxidatie activiteit, met methyl en benzyl viologeen als electron-acceptoren, Michaelis-Menten kinetiek.

In hoofdstuk 3 wordt beschreven hoe, door variatie van het milieu, door middel van zouten (hydrofiel) en hydrofobe stoffen zoals Me_2SO en ethyleen glycol,

en door oxidantia (zuurstof, ferricyanide, Cl_2Ind, (bi)sulfiet) de hydrogenase activiteit wordt beïnvloed. Uit de gegevens blijkt, dat, hoe chaotroper het anion van het zout, hoe groter de toename van de activiteit per mol zout. Me_2SO en ethyleen glycol remmen beide de waterstof productie activiteit, Me_2SO echter stimuleert de waterstof oxidatie activiteit, terwijl ethyleen glycol hierop geen effect heeft. Voorzichtige oxidatie van het gereduceerde enzym met (bi)sulfiet of Cl_2Ind verhoogt de activiteit van het enzym irreversibel met ongeveer 60%, dit in tegenstelling tot ferricyanide en zuurstof, deze oxidantia inactiveren het enzym. Dit 'irreversibel' geoxideerde enzym is stabieler tijdens opslag en is resistenter tegen de effecten van zuurstof, en Me_2SO, dan het gereduceerde enzym. Er is geen verschil in kinetische eigenschappen gevonden tussen deze vorm van het enzym en het niet geactiveerde enzym. Tevens worden de moeilijkheden beschreven om redox potentialen toe te schrijven aan EPR spectra van het hydrogenase in verschillende redox toestanden, zoals het effect van de temperatuur op de vergelijking van Nernst, de schijnbare pH, en op de 'midpoint' potentiaal van redox species.

In hoofdstuk 4 worden de theoretische aspecten beschreven om de waterstof productie activiteit als functie van de pH en redox potentiaal, spectrofotometrisch te bepalen in een serie gekoppelde redox reacties, tezamen met de beperkingen van deze methode. De waterstofproductie activiteit wordt bepaald uit de afname van de concentratie van de gereduceerde electron-donor, of uit de toename van de concentratie van de geoxideerde electron-donor. Deze verandering in concentratie van de gereduceerde of geoxideerde electron-donor is niet gelijk aan de hoeveelheid geproduceerde waterstof, maar is een maat voor deze hoeveelheid.

In hoofdstuk 5 wordt de methode, zoals beschreven in hoofdstuk 4, gebruikt om de effecten van de pH en redox potentiaal op de waterstof productie activiteit na te gaan. Het blijkt dat deze activiteit sterk afhangt van de pH en redox potentiaal. De waargenomen activiteitscurven als functie van de pH en redoxpotentiaal zijn onafhankelijk van het type electron-donor (methyl viologeen of flavodoxine) en vertegenwoordigen daardoor een eigenschap van het hydrogenase. Indien, bij een gegeven pH, de activiteiten worden beschouwd als maat voor de graad van reductie van het enzym, dan blijkt, dat het verband tussen activiteit en redox potentiaal opgevat mag worden als een n=2 type redox titratiecurve. Hierbij komt de schijnbare 'midpoint' potentiaal overeen met de potentiaal van de waterstof electrode bij die pH. Om de effecten van de electron-donor concentratie, de redoxpotentiaal en de pH te kunnen verklaren, werd het mechanisme zoals beschreven in hoofdstuk 2, enigszins aangepast, zonder de mathematische formulering van de reactievergelijking voor de waterstof productie activiteit te veranderen. Dit model

verklaart eveneens het 'verlies' aan electronen van het gereduceerde enzym tijdens EPR spectroscopie en geeft ook een verklaring voor de vorming van DD naar HD gedurende H_2-D_2O uitwisselings experimenten.

CURRICULUM VITAE

De auteur van dit proefschrift is geboren op 17 december 1949 te Gramsbergen. In 1968 behaalde hij het HBS-B diploma aan het Gemeentelijk Lyceum te Doetinchem. In hetzelfde jaar begon hij zijn studie aan de Landbouwhogeschool. In september 1972 behaalde hij het kandidaatsexamen Humane Voeding, natuurwetenschappelijke specialisatie, en in juni 1976 het kandidaatsexamen Moleculaire Wetenschappen, biologische specialisatie. Het doctoraalexamen Humane Voeding werd in juni 1976, het doctoraalexamen Moleculaire Wetenschappen in september 1976 behaald. De doctoraalvakken waren biochemie, humane voeding, en moleculaire biologie.

Het onderzoek voor dit proefschrift werd verricht op de afdeling Biochemie van de Landbouwhogeschool, waar hij van 1 augustus 1976 tot 1 augustus 1980 in dienst was bij de Stichting Scheikundig Onderzoek Nederland (S.O.N.) welke gesubsidieerd werd door de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.).