Translated from *Uspekhi Khimii*, **55**, 1524-1549 (1986) U.D.C. 547

Catalytic Properties of Hydrogenases

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The review considers the most general physicochemical properties of hydrogenases, the structure of their active centres, and the characteristics of the catalytic reactions in which they participate. It has been suggested that the hydrogenases be divided into one- and many-centre types in terms of the content of the iron-sulphur clusters in the protein molecule. The hydrogenases in these groups differ in their activity and physical and kinetic properties. Much attention has been devoted to the kinetic characteristics of hydrogenases, a scheme for the catalytic reaction has been deduced, and hypotheses have been put forward about the molecular mechanism of the action of these enzymes.
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I. INTRODUCTION

Processes involving the absorption and formation of molecular hydrogen in biological systems proceed as a rule with participation of a specific class of enzymes for which the trivial name "hydrogenases" has been proposed.[1] The general scientific and practical interest in these enzymes has been stimulated by a number of their features. Firstly, detailed study of the mechanism of the activation of hydrogen by biological catalysts makes possible a significant progress in our understanding of the physicochemical principles governing the enzyme catalysis of oxidation-reduction reactions as a whole. Secondly, the understanding of the mechanism of these enzymes should greatly facilitate the search for catalysts of the hydrogenation–dehydrogenation reactions of organic compounds which have found extensive practical applications. Furthermore, direct practical use of hydrogenases in reactions involving the oxidation or formation of hydrogen is of great interest. Thirdly, hydrogenases are terminal enzymes in the formation of hydrogen by micro-organisms,[2-4] which can be used, in particular for the utilisation of solar energy for the industrial preparation of hydrogen (the biophotolysis of water).[5] Furthermore, there is a possibility of creating model biochemical systems which effect the photodecomposition of water into hydrogen and oxygen.[6] Fourthly, the possibility of using enzymes in systems for the bioconversion of energy is extremely promising. The bioelectrocatalysis by hydrogenases can serve as the basis for the creation of fuel electrodes in electrochemical converters (biofuel cells).[7-11]

Hydrogenases are fairly common enzymes, which ensure the energy supply for many organisms by the oxidation of hydrogen. The metabolic pathways in micro-organisms exhibiting hydrogenase activity as well as the location and the functions of the hydrogenases themselves have now been thoroughly investigated. There are also numerous data on the biochemistry of these enzymes.[12-15] However, no general views about the catalytic properties of hydrogenases can be found in the literature and the kinetic characteristics of the individual enzymes are dealt with extremely incompletely and in a contradictory manner.

The aim of the present review is to give a systematic account of data on the catalytic functions of hydrogenases. The authors did not attempt to include all the available literature information about enzymes of this class and their aim was to seek the most general features.

II. CERTAIN PHYSICOCHEMICAL PROPERTIES OF IRON–SULPHUR PROTEINS

1. Hydrogenases as Iron–Sulphur Proteins

The first hypotheses concerning the presence of iron in the active centres of hydrogenases were based on the dependence of the activities of the enzymes on the concentration of iron in the culture medium of the micro-organism.[16] The direct radiochemical determination of the iron content in the fractions exhibiting hydrogenase activity at different stages of the purification of the preparation confirmed the presence of iron in the hydrogenases.[17] In addition, all the investigators, starting from 1943,[18] noted the influence of iron complexones on the activity of hydrogenases. Finally, direct determination by atomic-absorption spectroscopy demonstrated that the active centres of hydrogenases contain non-haem iron.[15]

At the same time a large series of studies devoted to the inhibitor analysis of hydrogenases with the aid of specific reagents for SH groups have been successful. It was established in the 1960's that *p*-chloromercuribenzoate, iodoacetamide, and mersalyl suppress hydrogenase activity.[19,20] However, the latter can be restored by incubation with reducing agents.[20-22] It has been shown[23] that, when the hydrogenase from *Clostridium pasterianum* is inhibited by mersalyl, the mercury ions interact with sulphur atoms, leading to the removal of iron from the active centre. The presence of the so-called "acid-labile" sulphur, which is evolved in the form of hydrogen sulphide on strong acidification of the preparations, has been established independently.[24-26]

At the beginning of the 1970's the presence of 12 iron atoms and 12 acid-labile sulphur atoms in the active centre of the enzyme was observed in a study of the hydrogenase from *Cl. pasterianum*.[24] Subsequent investigations confirmed that iron and sulphur are present in hydrogenases in equimolar amounts. The investigation of the optical spectra of hydrogenases made it possible to discover a chromophoric group, corresponding to an iron–sulphur cluster, in the active centre. The development of quantitative extrusion methods[27] made it possible to establish the identity of the structures of the clusters in the active centres of hydrogenases and low-molecular-weight iron–sulphur proteins. The structure and properties of the latter have been studied much more fully compared with hydrogenases. The characteristic features of the behaviour of the iron–sulphur clusters, which are extremely important for the analysis of the catalytic properties of hydrogenases, can be conveniently considered in relation to ferredoxins.

2. The Structure of the Active Centre and the Oxidation–Reduction Properties of Low-Molecular-Weight Iron–Sulphur Proteins

Vigorous study of iron–sulphur proteins began in 1962 when the ferredoxin from *Cl. pasterianum* was discovered.[28] One of the distinctive properties of the metalloproteins of this class is the presence of non-haem iron in the active centre.[29,30] Ferredoxins have a relatively low molecular weight (6000–30000).[29] They differ mainly in the structure of the active centre. The 1Fe proteins of rubredoxins contain one iron atom bound to four sulphur atoms in amino-acid cysteine residues. Apart from the two iron atoms, the 2Fe–2S ferredoxins contain two atoms of acid labile sulphur. The class of 4Fe–4S proteins is of greatest interest. It incorporates both ferredoxins and high potential iron proteins or HIPIP differing in the potentials of oxidation–reduction transitions. The proteins of the 8Fe–8S class and more complex ones do not have independent structures, their active centre being formed from the 2Fe–2S and 4Fe–4S clusters.

The iron–sulphur proteins have come to be divided into two groups in terms of the oxidation-reduction potential. The class incorporating the 2Fe–2S and 4Fe–4S proteins, whose potentials are negative (−0.3–−0.4 V), is largest. Here and henceforth the potential is quoted relative to the normal hydrogen electrode. The 4Fe–4S proteins with potentials of about +0.3 V belong exclusively to the group of high-potential proteins. In order to explain the above difference, the hypothesis of "three states" has been put forward,[31] according to which the transitions in ferredoxins and HIPIP take place between different electronic states of the clusters determined by the ratio of the Fe^{2+} and Fe^{3+} atoms. The discovery of superoxidised ferredoxins (Fd_{S-ox}) and super-reduced HIPIP (HP_{S-red}) suggested the model of "three states" and the final scheme for the oxidation-reduction transitions in the 4Fe–4S proteins:[32]

$$\begin{array}{ccccc} 4Fe^{2+} & Fe^{3+}+3Fe & 2Fe^{3+}+2Fe^{2+} & 3Fe^{3+}+Fe^{2+} & \\ [Fe_4S]^0 & \leftrightarrow [Fe_4S_4]^{1+} & \leftrightarrow [Fe_4S_4]^{2+} & \leftrightarrow [Fe_4S_4]^{3+} & \\ & Fd_{red} & Fd_{ox} & Fd_{s-ox} & \\ & HP_{s-red} & HP_{red} & HP_{ox} & . \end{array}$$

Here and henceforth the charge on the cluster corresponds to the sum of the charges on the iron and acid–labile sulphur atoms.

The study of the physical properties also led to the elucidation of the nature of the ferredoxin transition in the 2Fe–2S proteins. It was established that in the oxidised cluster both iron atoms are in the +3 oxidation state; the reduction of the cluster leads to the transition of one of the atoms to a bivalent state.[33]

The fact that the iron–sulphur cluster constitutes a single electronic structure is remarkable. Numerous investigations have shown that the iron atoms within the cluster are indistinguishable.[30] In order to account for this effect, the hypothesis of the "antiferromagnetic interaction", based on the valence bond method, has been put forward.[33]

Many investigators (for example, Sweeney and Rabinowitz[34]) have pointed out that the spectroscopic characteristics of the 2Fe–2S and 4Fe–4S clusters are extremely similar and sometimes it is impossible to identify the type of cluster. This is caused by the binding of other chromophores and frequently by the influence of the polypeptide chain itself. These difficulties can be overcome by applying the magnetic circular dichroism method[35] and the effect of the linear electric field, which alters the EPR spectrum.[36] However, the simplest method consists in the study of the EPR spectra in 80% dimethyl sulphoxide at different temperatures.[37]

The number of iron atoms in the active centres of hydrogenases is a multiple of four. The application of the quantitative extrusion method to the 12Fe hydrogenases from *Cl. pasterianum*[27] and *Desulfovibrio vulgaris*[38] demonstrated the presence of only the 4Fe–4S clusters in the active centre. In addition there exists a whole series of enzymes containing one 4Fe–4S cluster per protein molecule. However, two 2Fe–2S clusters were detected together with two 4Fe–4S clusters in a study of the EPR spectra of the 12Fe hydrogenases from *Alcaligenes eutrophus* H16.[39,40] Such detailed studies have not been made for other multicluster hydrogenases, but the presence of the 4Fe–4S cluster in the active centre of the enzyme is apparently always essential for hydrogen catalysis. We shall consider briefly the properties of such clusters.

3. The Physical Properties of the 4Fe–4S clusters in Low-Molecular-Weight Proteins

(a) *Optical Properties.* The absorption spectra in the visible region of the iron–sulphur proteins containing clusters in the same oxidation states are identical. The absorption at 400 nm diminishes in the sequence

$$[Fe_4S_4]^{3+} > [Fe_4S_4]^{2+} > [Fe_4S_4]^{1+}.$$

The ratio of the absorptions at 400 and 280 nm is a measure of the ratio of the chromophore and the peptide and is usually employed as an indicator of the purity of the iron–sulphur protein.[34]

(b) *Magnetic Properties.* An even number of unpaired electrons in the cluster predetermines the absence of EPR signals. Thus the $[Fe_4S_4]^{2+}$ state is undetectable by EPR.

The $[Fe_4S_4]^{+}$ clusters have a characteristic EPR spectrum which depends relatively weakly on the polypeptide environment. An axial or orthorhombic signal is observed and the g-factor is about 1.96. If one excludes the influence of the peptide by recording the spectrum in 80% dimethyl sulphoxide (DMSO),[27] one can obtain an axial signal with the parameters $g_\perp = 1.94$ and $g_\parallel = 2.05$. Ferredoxins

containing two and more iron–sulphur clusters in the $[Fe_4S_4]^+$ state give rise to a more complex EPR signal, which can be accounted for by spin–spin coupling.[34]

The EPR spectra of the $[Fe_4S_4]^{3+}$ clusters are much more sensitive to the polypeptide environment. They are characterised by the factor $g > 2$ (one unpaired electron per four iron atoms).

(c) *The influence of the protein globule.* The protein globule can have a twofold effect on the properties of the cluster. Firstly, it influences on the choice of the optimum oxidation-reduction transitions (ferredoxins and HIPIP). Secondly, depending on the protein, a fairly wide variation of the oxidation-reduction potential is possible for the same reaction. It has been noted[34] that the potentials of the ferredoxin transition $[Fe_4S_4]^{2+} \leftrightarrow [Fe_4S_4]^+$ vary within the limits of 650 mV, while those of the HIPIP transition $[Fe_4S_4]^{3+} \leftrightarrow [Fe_4S_4]^{2+}$ vary up to 750 mV. The conformational changes of the ferredoxin from *C. acidiurici* in the course of the reduction have been recorded.[41] Carter et al. observed in their study of the high-potential proteins from *Chromatium sp.* by X-ray diffraction[42] that the symmetry of the iron–sulphur cluster changes in the oxidation-reduction transition. The protein globule can stabilise or destabilise a particular state of the cluster by its conformational influence, by acting upon the choice of the optimum transition, on the one hand, and by shifting the thermodynamic equilibrium of the oxidation-reduction reaction and hence its potential, on the other.

It has also been stated in the literature that two 4Fe + 4S clusters, greatly differing in the potential of the same electronic transition $[Fe_4S_4]^{3+} \leftrightarrow [Fe_4S_4]^{2+}$ may be present in the iron–sulphur proteins. Ferredoxin I from *Azotobacter vinelandii*[43] has potentials of −420 and +320 mV, the corresponding potentials of ferredoxin IV from *Rhodospirillium rubrum*[44] are respectively −380 and +355 mV, and those of ferredoxin I from *Myobacterium flavum*[45] are −420 and +230 mV.

The ability of the iron–sulphur protein to be degraded by atmospheric oxygen can be accounted for mainly by the lability of the cluster. It has been shown[46] that the interaction of the 4Fe–4S cluster with O_2 leads to the transfer of one iron atom to the solution. The discovery of clusters of the type 3Fe–*n*S confirms this conclusion.

4. The 3Fe–*n*S Clusters

The 3Fe–*n*S cluster was first detected in ferredoxin II from *Desulfovibrio gigas*.[47] Then it was shown that the ferredoxin from *Cl. pasterianum* is also converted into a form containing a similar cluster on oxidation with hexacyanoferrate(III).[48]

On reconstructing ferredoxin II from *D. gigas* containing initially one each of the 4Fe–4S and 3Fe–*n*S clusters,[49] it was found that an excess of bivalent iron and S^{2-} leads to the formation of clusters of the first type exclusively. An increase of ionic strength or a decrease of the iron concentration in solution has the same effect involving the appearance of a considerable proportion of the 3Fe–*n*S cluster. Furthermore, there is a possibility of the direct interconversion 4Fe–4S ↔ 3Fe–*n*S.[49] Under severe oxidation conditions [in the presence of hexacyanoferrate(III) and oxygen] the 4Fe–4S clusters are converted into the 3Fe–*n*S form, liberating one bivalent iron atom to the solution. It was found possible to carry out also the reverse process under oxidative conditions, the 3Fe–*n*S clusters are capable of being converted into 4Fe–4S under the influence of dithiothreitol and iron salts.

III. PHYSICOCHEMICAL PROPERTIES OF HYDROGENASES

1. Oxidation-reduction Properties

Hydrogenases are capable of both activating molecular hydrogen and of catalysing its evolution. Numerous experiments designed to isolate and store enzymes under various conditions have shown that, under the influence of atmospheric oxygen, the hydrogenase is converted into an inactive form, which, however, may be reactivated by incubation with reductants. It is of interest to determine the influence of the oxidation-reduction state of the enzyme on its activity.

However, serious errors have been made in some studies of this phenomenon. In the course of the measurement of the activity of the hydrogenase on the basis of the rate of evolution of H_2, a number of workers[50–53] determined in essence the potential dependence of the hydrogen electrode (see, for example, Damaskin and Petrii[54]), i.e. the potential of the equilibrium evolution of hydrogen from water. Nevertheless, the loss of activity of the enzyme at potentials less than −480 mV under strongly reductive conditions has been reliably established.[51]

The oxidation-reduction titration of the hydrogenase from *Thiocapsa roseopersicina* using various electron donors made it possible to determine the activation potential ($E = -276 \pm 8$ mV).[55]

Although reduction promotes the activation of hydrogenases, nevertheless the direct reduction of the active centre also leads to the loss of activity. There is no doubt about the inhibiting effect of reducing agents, especially sodium dithionite, on the activity of hydrogenases.[13] Together with the irreversible loss of activity,[56] many investigators point out either a weak effect or the possibility of reactivation[20,55,57,58]). The inactivation of the hydrogenase from *Cl. pasterianum* at potentials below −480 mV was noted above.[51] At low concentrations, sodium dithionite does not affect the rate of evolution of hydrogen which takes place with participation of the enzyme from *D. vulgaris*;[59] an increase of the concentration of the reductant leads to a fall of the activity of the hydrogenase to zero.

Together with the usual "two-sided" hydrogenases, there exist a number of hydrogenases capable mainly of absorbing hydrogen. They are all located in the cells of microorganisms together with nitrogenase, which is apparently a factor determining their physiological function. The activity of the "one-sided" hydrogenases as regards the evolution of hydrogen is usually lower by two orders of magnitude than the activity measured on the basis of the absorption of H_2 under the same conditions. The rate of evolution for *Rhodopseudomonas capsulata*,[60] *Cl. pasterianum*,[52] and *Rhizobium japonicum*[61] is 1–3% of the rate of absorption of H_2; the corresponding activities of the "one-sided" hydrogenase from *Azotobacter chroococcum*[62] differ by a factor of 200.

It has been suggested[63] that the reduction of "one-sided" hydrogenases takes place even in the positive range of potentials relative to the hydrogen electrode in the same solution. Thus, under conditions thermodynamically favourable for the evolution of hydrogen, the hydrogenase becomes inactive. The inactivating reduction potential for the enzyme from *Rh. capsulata*[63] at pH 7.0 was found to be 340 mV. This quantity depends on the pH: $\partial E/\partial \mathrm{pH} \approx -30$ mV. At pH 5.0 the rates of evolution and absorption of hydrogen are approximately the same.

2. Optical Properties

Hydrogenases have optical spectra characteristic of iron–sulphur proteins. An absorption maximum at a wavelength of approximately 400 nm is observed in the visible region. The extinction coefficients of hydrogenases calculated per iron atom are usually 4000 litre mol^{-1} cm^{-1}, but may deviate significantly from this value.[14,15] When hydrogenases are reduced to the active state by various agents (including sodium dithionite and hydrogen), the absorption at 400 nm diminishes by 10–20%.[14]

3. EPR Spectra

Hydrogenases give rise to EPR signals characteristic of iron–sulhpur proteins. In terms of the character of the changes in the EPR spectra during the oxidation–reduction of the active centre, hydrogenases can be divided into two groups. The one-centre enzymes, i.e. those containing one 4Fe–4S cluster, have in the oxidised state an EPR spectrum with $g > 2$, which is extremely similar to the spectrum of the high-potential proteins in the oxidised state, i.e. the spectrum of the $[Fe_4S_4]^{3+}$ cluster. When these hydrogenases are reduced, the EPR signal vanishes. Consequently the oxidation-reduction transition in the enzymes of this group corresponds to the transformation $[Fe_4S_4]^{3+} \leftrightarrow [Fe_4S_4]^{2+}$. Such data have been obtained for the hydrogenases from *Th. roseopersicina* and *Rh. capsulata*,[63] *Rhodospirillium rubrum*,[64,66] *Chromatium vinosum*,[67] and *Chromatium sp.*[68] The authors of the last investigation noted, like Zorin et al,[63] that carbon monoxide does not affect the EPR signals of hydrogenases either in the oxidised or reduced states.

Many-centre hydrogenases (containing more than four iron atoms in the active centre) behave in a significantly different manner. Whereas in the oxidised state they too give rise to EPR signals resembling the $[Fe_4S_4]^{3+}$ signal, after reduction, spectra characteristic of reduced ferredoxins are observed, i.e. characteristic of the state of the $[Fe_4S_4]^{+}$ cluster with the typical $g = 1.94$. The hydrogenase from *Megasphaera elsdenii*, containing three clusters per molecule, has an EPR signal with $g > 2$ in the oxidised state, which is somewhat atypical for HIPIP; when it is reduced, the characteristic ferredoxin spectrum is manifested.[69] Conversely, the hydrogenase from *Cl. pasterianum*, containing 12 iron atoms in the centre, is characterised in the reduced state by the presence of an additional "extraordinary" signal in the ferredoxin spectrum.[70] The authors believe that in the oxidised state only the $[Fe_4S_4]^{3+}$ signal is observed. The number of 4Fe–4S clusters in the hydrogenase from *D. vulgaris* is three.[38,71,72] The EPR spectra of this hydrogenase are typical for many-centre hydrogenases.[38,73]

The oxidation-reduction titration of the enzyme from *A. eutrophus* H16, containing 12 iron atoms per molecule, at different temperatures in 80% DMSO made it possible to distinguish the EPR signals of the two 4Fe–4S and two 2Fe–2S centres.[39,40] The electronic transitions take place at potentials of −445 and −325 mV respectively.

Thus the one-centre hydrogenases in the oxidised state contain the $[Fe_4S_4]^{3+}$ cluster. On reduction, they pass to an active state corresponding to the $[Fe_4S_4]^{2+}$ form. The oxidation-reduction potentials of the above electronic transitions lie mainly in the negative region (for example, at −276 mV for *Th. roseopersicina*[55]). Consequently, the hydrogenases of this type have the properties of low-potential HIPIP proteins. Re-reduction leads apparently to a transition to the $[Fe_4S_4]^{+}$ state in which the enzymes are inactive.

The situation is more complex for many-centre hydrogenases. In the reduced state the EPR spectra resemble those of the $[Fe_4S_4]^{+}$ clusters, while in the oxidised state they resemble the spectra of $[Fe_4S_4]^{3+}$. Chen suggested that in the hydrogenase from *Cl. pasterianum*,[52] containing three 4Fe–4S centres,[27] there are two $[Fe_4S_4]^{2+}$ clusters and one $[Fe_4S_4]^{+}$ cluster in the oxidised state and two $[Fe_4S_4]^{+}$ clusters and one $[Fe_4S_4]^{2+}$ cluster in the reduced state. However, the simultaneous transfer of three electrons (in the potential range 50–60 mV) is possible only for vigorously isolated clusters, while the available data indicate the interaction of the latter. It has been shown[38] that the three-centre hydrogenase (incorporating three clusters of the 4Fe–4S type) from *D. vulgaris* contains no $[Fe_4S_4]^{3+}$ clusters in the oxidised state. The form of the spectrum of this hydrogenase, resembling the HIPIP spectrum, is apparently due to the spin–spin coupling of the clusters in other oxidation states, namely $[Fe_4S_4]^{2+}$ and $[Fe_4S_4]^{+}$. Unfortunately, it was not possible to determine the electronic structure of the active centre of the hydrogenase in greater detail.

4. The Relation between the Physical and Catalytic Properties of Hydrogenases

It is of interest to compare not only the physical but also the catalytic properties of the one-centre and many-centre hydrogenases. The Table shows that the activity of the latter, as regards the absorption and evolution of hydrogen, is on average higher by two orders of magnitude than that of the one-centre enzymes. It will be shown below that the cleavage of the hydrogen molecule and the proton exchange with the solvent are not rate-limiting in the H_2 activation and evolution reactions.

On this basis, one can put forward two hypotheses concerning the role of the iron–sulphur clusters in catalysis. According to the first, the cluster is an electronic structure stabilising the transition state and hence reducing the height of the activation barrier (see, for example, Berezin and Martinek[77]). The hydrogen oxidation pathway via the intermediate formation of the charged forms of the enzyme (E^- and E^{2-}), of the kind suggested, for example, by Chen[52] is unlikely. On the other hand, the undue stabilisation of the intermediate complex leads to an increase of the height of the activation barrier.[77]

According to the second hypothesis, the change in the symmetry of the iron–sulphur clusters in oxidation–reduction transitions (see above) can entail an enforced stabilisation or destabilisation of one of the electronic states under the influence of the thermal movement of the protein globule, which decreases the activation energy via the entropy term.

However, the presence of iron–sulphur clusters in the protein molecule is insufficient for the manifestation of the hydrogenase activity. In contrast to one-centre hydrogenases, the low-potential HIPIP catalyse neither the evolution nor the absorption of hydrogen. An example has been provided by the ferredoxins from *D. gigas*[78] and *M. flavum*,[45] containing four iron atoms per molecule and having oxidation-reduction potentials of −130 and −420 mV respectively, as well as the 6Fe ferredoxins from *T. thermophilis*,[79] whose potential is −250 mV.

5. The Role of Nickel in the Hydrogenase Catalysis

The studies in recent years have shown that the presence of nickel in the active centre is extremely important for the manifestation of the hydrogenase activity. This conclusion originated from experiments concerning the effect of the nickel content of the culture medium on the synthesis of hydrogenases during the growth of the micro-organism. It has been shown[80] that the addition of EDTA (at a concentration of 150 μM) to the propagation media of three different cultures (*Azospirillium brasilense*, *A. lipoberum*, and *Derxia gummosa*) suppresses the synthesis of the hydrogenase without influencing significantly the activity and only the introduction of catalytic amounts of bivalent nickel ions (10 μM) makes it possible to abolish the inhibiting effect of EDTA. A correlation has been found[81] between the absorption of radioactive $^{63}Ni^{2+}$ by the cells of *Azotobacter chroococcum* and their hydrogenase activity. The addition of bivalent nickel ions to the culture medium of *Chromatium vinosum*[82] increases the activity of the hydrogenase by a factor of 3–6 without affecting the rate of growth of the micro-organism.

It has been observed that, during the purification of the hydrogenase from *Vibrio succinogenes*,[83] nickel is washed out of the preparation. The authors state that the dependence of the activity of the enzyme on the Ni^{2+} concentration is represented by a straight line passing through the origin of coordinates. Admittedly, individual points on this straight line corresponded to specimens of the enzymes with different degrees of purity.

The involvement of nickel in the hydrogenase catalysis has been confirmed by physicochemical data. The EPR spectrum of the enzyme from *Chromatium vinosum*[82,84,85] shows that the 4Fe–4S cluster interacts with the nickel centre. The effect of the latter is not manifested in a hydrogen atmosphere. The unperturbed spectrum of the cluster can be observed when it is converted into the 3Fe–*n*S form, which, as stated by the authors, is catalytically inactive. Nickel has also been detected by EPR in the hydrogenases from *M. thermoautotrophicum*,[86] *D. gigas*,[87] and *D. vulgaris*.[38]

The EPR spectra of the nickel present in the hydrogenase from *D. gigas* have been studied for different oxidation-reduction states of the enzyme.[87–89] It has been shown that the nickel centre is capable of entering into a one-electron exchange reaction, altering the oxidation state. The potential of this transition is −220 mV at pH 8.5 [88] and depends greatly on the pH ($\partial E/\partial pH = -60$ mV).[89] Thus the reaction is proton-dependent, the Ni^{3+} form being singly protonated:

$$Ni^{3+}+H^{+}+\bar{e}\rightleftharpoons Ni^{2+}H^{+} \qquad (1)$$

Bearing in mind the character of the oxidation-reduction transition (1), we normalise the potentials at pH 7.0. Very close values are obtained for the many-centre enzyme from *D. gigas*[88,89] and the one-centre enzyme from *Chromatium vinosum*[85] (−140 and −150 mV respectively). In the active state of the hydrogenase (in the vicinity of the hydrogen electrode potential) the nickel exists in a bivalent state.

It has been suggested[85] that the nickel centre is the primary acceptor of the hydrogen molecule. It is believed that the Ni atom has an octahedral environment with five occupied coordination centres and one centre capable of being substituted. According to EPR data the minimum distance between the iron and nickel atoms in the hydrogenase from *Chromatium vinosum* does not exceed 12 Å.

Comparative characteristics of the physical and catalytic properties of certain hydrogenases.

Source of hydrogenase	Content per molecule		Molecular weight	Type of EPR spectra		Activity, μmol H_2 min^{-1} mg^{-1}		Substrate	Refs.
	Fe	S		oxidised state	reduced state	absorption of H_2	evolution of H_2		
Th. roseopersicina	4	4	68 000 (1×47 000, 1×25 000)*	HIPIP	—	1.2 19.0	5.0 —	MV BV	[55]
Rh. capsulata	4	4	73 000	HIPIP	—	0.075 0.83 1.1	0.012 — —	MV BV MC	[74,75]
Rh. rubrum	4	4	67 000	HIPIP	—	0.26 1.8 2.5	26.0 7.0	MV BV MC	[64,65]
Chromatium vinosum	4	4	61 000–63 000	HIPIP	—	—	425	MV	[67]
Chromatium sp.	4	4	98 000 (2×50 000)*	HIPIP	—	210 78 —	— — 39	MC BV MV	[22] [68]
Megasphaera elsdenii	12	12	50 000	HIPIP (atypical)	— g=1.94	— —	630 8400**	MV	[69,71,76]
Cl. pasterianum	12	12	60 000	HIPIP	Fd spectrum; $g\cong 2.0$	1660 — —	317 3960 4000** 5000**	MV MV+Fd MV MV+Fd	[24] [52]
D. vulgaris	12	12	50 000	$g\cong 2.0$	$g_{\perp}$=1.86 $g_{\parallel}$=2.03	4600** 5000** — —	— — 3800 10 400**	MV BV MV	[72] [71]

Notation: MV = methylviologen; BV = benzylviologen; MB = Methylene Blue.

*The subunit composition is given in brackets.
**The maximum reaction rates are quoted.

6. Characteristics of the Catalysis by the Hydrogenase from *A. eutrophus*

The hydrogenase from *A. eutrophus* includes yet another group involved in hydrogen catalysis, namely the flavin mononucleotide (FMN),[57] which is responsible for its NADH-dehydrogenase activity. The enzyme molecule (molecular weight 180000–200000) contains 12 atoms of iron and acid-labile sulphur each, combined in two 4Fe–4S and two 2Fe-2S clusters,[39,40] and two FMN molecules.[90] The enzyme consists of four subunits, the hydrogenase activity being manifested solely by the complete molecule,[91] while the NADH-dehydrogenase activity is retained in subunits with molecular weights up to 60000. The enzyme from *A. eutrophus* has unique properties, behaving simultaneously as a hydrogenase and a NADH-dehydrogenase. During the oxidation of hydrogen, the electron density acceptor (via intermolecular electron transfer), is FMN which is capable of reacting subsequently with NAD, reducing it to NADH. It has been shown[92] that methylviologen is able to "intercept" the electron density on the way to the FMN molecule. We may note that the reduced FMN is weakly oxidised by oxygen, like NADH. As a result, NAD should react with FMNH even under aerobic conditions. Indeed, the rate of reduction of NAD by hydrogen, catalysed by the hydrogenase from *A. eutrophus* Z1, is virtually the same in an open cuvette and under anaerobic conditions.[93] The competitive inhibition of the enzyme from *A. eutrophus* H16 [94] by oxygen with respect to H_2 has been noted. In the subsequent stages the electron density remains inaccessible to O_2. Thus the unique characteristic of the hydrogenase of *A. eutrophus* consists in the fact that this enzyme is capable of oxidising hydrogen with subsequent reduction of the acceptor, which has a low negative potential, almost independently of the presence of oxygen. This is so far impossible on any other catalyst.

IV. THE INFLUENCE OF VARIOUS EFFECTS ON THE ACTIVITY OF HYDROGENASES

1. The Role of the Ionic Strength of the Solution

Contradictory data concerning the influence of the ionic strength of the solution on the activity of hydrogenases have been quoted in the literature. For example, a high ionic strength has an inhibiting effect on the enzymes from *Paracoccus denitrificans*[95,96] and *D. vulgaris*.[71] The activity of the hydrogenase from *Chromatium vinosum*[67] falls appreciably in the presence of cobalt sulphate and chloride. The enzymes from *Chromatium sp.*[22] are inhibited by heavy metal ions.

On the other hand, the activity of the hydrogenase from *Megasphaera elsdenii*[69,71] increases with increase of the ionic strength of the solution. The stabilising effect of chlorides at concentrations in excess of 0.7 M on the enzyme from *Rh. rubrum* has been noted.[97] The hydrogenase from *Spirilina maxima*[98] is inactivated by copper and zinc ions, but the presence of iron, manganese, magnesium, and calcium leads to a slight activation.

The contradictory nature of the above data gives some indication of the mechanism of the effects. The ionic strength apparently acts on the protein globule. Evidently the proteins of even a single micro-organism, located in different places within the cell, can show significant differences. In conclusion, we may mention the retention of the activity of the hydrogenase from *D. vulgaris*[99] in the lyophilised state.

2. The Effect of Iron Complexones

At the beginning of the 1970's many workers observed a strong inhibition of the activity of hydrogenases by iron complexones: αα′-bipyridyl, *o*-phenanthroline, EDTA, CN^-, and N_3^- (see, for example, Bone[20] and Hashke and Campbell[100]). However, it was found subsequently that the above reagents have only a weak effect on the activity of the enzymes from *Chromatium sp.*[22] and *D. vulgaris*.[101] The hydrogenases from *Anabaena cylindrica*[102] and *E. coli*[103] are not inhibited by αα′-bipyridyl, while the enzyme from *Chromatium vinosum*[67] is unaffected by CN^- and N_3^-. Furthermore, EDTA, αα′-bipyridyl, and *o*-phenanthroline have a prolonged effect against the inactivation of the hydrogenase from *Cl. pasterianum* by oxygen,[104] while CN^-, N_3^-, and F^- do not affect its activity at concentrations up to 10^{-2} M.[105]

Although it has been recognised that the above iron complexones are not inhibitors of hydrogenases,[14,15] studies are still being published in which a decrease of their activity in the presence of EDTA, CN^-, and N_3^- has been noted.[98] The effect of these reagents is apparently associated with the partial degradation of the iron–sulphur cluster and should therefore depend on the stability of the latter. Indeed, the stability of the hydrogenase from *A. cylindrica* with respect to inactivation by oxygen has been noted in a study[102] where the absence of an effect by αα′-bipyridyl on the activity of the enzyme was observed. The unusual stability of the clusters from *Chromatium sp.* with respect to extrusion[106] agrees well with their insensitivity to iron complexones.[22]

The effect of carbon monoxide should be considered specially. The inhibiting action of CO has been found in all studies in which its influence on the activity of hydrogenases has been investigated. It is difficult to suppose that this entails any changes in the electronic structure of the iron–sulphur cluster, because CO shows no effect on its EPR spectrum.[67,68] It has been established that one CO molecule is adsorbed on the active centre of the enzyme.[67,102] In the hydrogen oxidation reaction the inhibition by carbon monoxide is competitive with respect to H_2 [105,107–109] and non-competitive with respect to the electron acceptor.[107,108] Thus, within the framework of the hypothesis that the nickel centre is an acceptor of the H_2 molecule while the iron–sulphur cluster stabilises the intermediate state in electron transfer, the inhibiting effect of CO can be readily accounted for. Carbon monoxide evidently blocks the free coordination site in the nickel centre which is inaccessible to larger complexones. The hydrogen molecule is also adsorbed at this coordination site.

3. The Mechanisms of the Inactivation of Hydrogenases by Oxygen

The high sensitivity of hydrogenases to oxygen is well known. Attempts have therefore been sometimes made to extract the enzymes from strict anaerobes under the conditions of the controlled absence of O_2.[24,100,110] Subsequent studies led to the abandonment of this complication in biological techniques. Two mechanisms for the action of oxygen on the activity of hydrogenases have been discovered: (1) reversible oxidation of the active centre in which the possibility of its reactivation by a reductant remains; (2) slow irreversible inactivation. The occurrence of the latter has been demonstrated.[111]

The slow irreversible inactivation in air is characteristic of all hydrogenases. Nevertheless the enzymes of *A.eutrophus* Z1 proved to be more stable on storage in air than in an atmosphere of a pure inert gas or hydrogen.[93] The stabilising effect of oxidants has also been noted in other studies.[39,40] The kinetics of the irreversible inactivation of hydrogenases by oxygen indicate a distinct two-phase character of the process,[93,112,114] the reduced form of the enzyme being labile and the oxidised form stable.[93] The conclusion that the oxidised hydrogenase is more resistant to O_2 than the reduced form has been confirmed also by other data.[39,71,113]

The attempts to stabilise hydrogenases in air are associated in the first place with immobilisation. The two-phase character of the inactivation of the enzyme from *Cl.pasterianum*[114] persisted also after immobilisation on glass, but its stabilisation was then achieved. The degree of blocking of the active centre of the enzyme, determined from the ratio of its activity in the presence of low-molecular-weight (methylviologen) and high-molecular-weight (ferredoxin) electron transferring agents, is satisfactorily correlated with the stabilising effect.

A novel method for protecting against O_2 has been proposed.[115] By immobilising the protein on the charged surface of a polyanionic adsorbent, it is possible to create a microenvironment in which "gasses are salted out". The adsorption of the hydrogenase from *Cl.pasterianum* on DEAE-cellulose or Dowex 1-X2 increased the stability of the enzyme in air by a factor of 20–25. It was noted that at a low ionic strength hydrogen was also no longer in contact with the hydrogenase. Consequently, when this method is used, the reaction can be effected only in the direction of H_2 evolution.

However, another stabilisation procedure, which throws light on the mechanism of the inactivating effect of oxygen, is the most interesting. It has been found that iron complexones (EDTA, αα′-bipyridyl, *o*-phenanthroline, and Chelex-100) at concentrations in the range 0.01–0.5 M have a prolonged effect on the hydrogenase from *Cl.pasterianum*,[104] while αα′-bipyridyl and benzylviologen[103] lower the rate of the oxygen inactivation of the enzyme from *E.coli*. Finally, the protecting effect of carbon monoxide against the action of O_2 has been observed and it has been shown that the hydrogenase from *Chlamydomonas reinhardi* reacts with one oxygen molecule at the rate-limiting stage of the inactivation process.[116]

It has been reported that the reduction of the reduced enzyme from *A.eutrophus* H16 with O_2 leads to the appearance in solution of the superoxide radical-ions O_2^-.[117] Like hydrogen peroxide, the latter inactivate the hydrogenases from *E.coli*, *Cl.pasterianum*,[103] *Th.roseopersicina*, and *Rh.capsulata*[118] more strongly than does molecular oxygen. It may be that this is one of the factors determining the relative lability of the reduced forms of the enzymes. However, the superoxide radical-ions had no effect on the hydrogenase from *A.eutrophus H*16.[94]

The degradation of the iron–sulphur clusters in hydrogenases by oxygen is to some extent analogous to the interconversion in ferredoxins. The 4Fe–4S cluster is capable of being converted into the 3Fe–*n*S form with liberation of an iron atom into the solution, especially under the influence of O_2.[49] The 3Fe–*n*S cluster is believed to be inactive in hydrogen catalysis.[82,84,85,87] Furthermore, in studies[84,85] of the EPR spectra of the hydrogenase from *Chromatium vinosum* it was found that the interconversion interferes with the electronic interaction of the iron–sulphur and nickel centres, which is also important for catalysis. It has been suggested that the iron atom nearest to nickel is liberated from the cluster. However, the attempts to reconstruct the hydrogenases by analogy with ferredoxins[49] led to the recovery of only traces of activity for *D.vulgaris*[119] and were unsuccessful in many instances. However, there exists an indirect confirmation of the proposed hypothesis. The fact that bivalent iron ions stabilise the hydrogenase from *Azotobacter chroococcum* and prevent the loss of its activity[62] can be interpreted as a consequence of their effect on the kinetics of the degradation of the 4Fe–4S cluster, which takes place with the evolution of a bivalent ion into the solution.

Another consequence of the effect of oxygen on hydrogenases is rapid reversible inhibition. As already stated, the enzymes isolated and stored in air are in an inactive state. They can be reactivated by adding reducing agents. Furthermore, in the oxidation of H_2 or in deuterium exchange there is a possibility of an autocatalytic acceleration of the reaction after an induction period.[20,57,120–122]

The phenomenon of autocatalysis has been studied in detail in relation to the enzymes from *Th.roseopersicina*[120] and *A.eutrophus*.[93] It was shown that the induction period decreases or is completely eliminated after the addition of reducing agents, for example sodium dithionite, or very small amounts of the product, i.e. the reduced form of the electron acceptor. The lag period is shortened also after increasing the concentrations of the enzyme[93,120,122,123] and the electron acceptor,[120] which is extremely characteristic of reactions under autocatalytic conditions.[120] However, the opposite variation has been observed in the reduction of NAD by hydrogen, which is catalysed by the hydrogenase from *A.eutrophus* Z1: the induction period increases with increase of the NAD concentration.[93] We may recall that NADH inhibits the hydrogenase from *A.eutrophus* Z1 [91] by cleaving it into subunits. The superposition of the inhibition by the product does in fact lead to this type of extraordinary variation.

The question of the mechanism of the activation of hydrogenases is of fundamental importance: does it occur under the influence of hydrogen, in the reduced electron acceptor, or as a result of a proenzyme–enzyme interaction. Data on autocatalysis have shown that there is no direct activation of the enzyme by hydrogen. In the oxidation of hydrogen by methylviologen,[120] the hydrogenase from *Th.roseopersicina* is activated solely by the interaction of the inactive enzyme with the reduced methylviologen. However, one should note that the concentration of MV^{2+} in this reaction was much higher than in the enzyme concentration.

The study of the deuterium exchange involving the hydrogenase from *A.eutrophus H*16 [123] showed that in solution the enzyme can be activated also in the absence of an electron transferring agent, while in the immobilised state the free electron transferring agent is essential for this purpose in the absence of the enzyme–enzyme interaction. The impossibility of activating the enzyme directly by hydrogen has also been confirmed in another study.[92] Consequently the activity of the hydrogenase irreversibly inactivated by oxygen can be restored under autocatalytic conditions via the interaction either with the reduced form of the electron transferring agent or with the molecules of the active enzyme.

The "oxygenation" hypothesis was proposed in 1954, according to which[122] the reversible inactivating effect of oxygen reduces to the formation of a complex and not to an oxidation-reduction reaction:

$$E + O_2 \rightleftarrows EO_2,$$

where EO_2 and E are the inactive and active forms of the

enzyme. Oxygen can be evolved from the active centre of the enzyme as a result of dissociation. The authors believe[122] that the hydrogenase is activated in this way.

Since this theory has gained fairly wide currency,[13,15] it is essential to consider it in greater detail. The authors[122] believe that the correlation between the "oxygen-absorption" activity of the reductant and the decrease of the induction period for the hydrogenase from *P. vulgaris* constitute proof of "oxygenation". However, the lag period in the oxidation of hydrogen catalysed by the hydrogenase from *A. eutrophus* is eliminated both as a result of the glucose oxidase reaction (glucose + glucose oxidase),[121] which the above authors[122] regard as one of the best "deoxygenants", and after the addition of very small amounts of NADH (10^{-4} M),[93] which interact very weakly with O_2. We may note the potential of the oxidation of glucose to gluconate[124] is -0.42 V.[7] Oxygen may not be the only oxidant in this reaction. Finally, data for the oxidation-reduction transitions in the active centres of hydrogenases on activation (see above) are entirely inconsistent with the "oxygenation" hypothesis.

Autocatalysis takes place owing to the presence of micro-amounts of the active form of the enzyme, which are inevitably present even at high positive potentials. In the initial stages of the reaction, oxygen is present in solution, so that O_2 reacts with the reduced forms of the enzyme and the electron transferring agent. A dependence on the oxygen concentration appears as a result and this has been recorded.[122] The competition between autocatalysis and the inhibition of the reaction by oxygen leads to the appearance, in the general case, of more complex dependences of the rate of acceleration on the specific activity of the hydrogenase, the oxidation-reduction potentials of the enzyme and the electron acceptor, and the concentrations of all the components, including O_2. The existence of a threshhold concentration of the enzyme has been indicated.[123]

4. The Effect of the pH of the Medium on Catalysis by Hydrogenases

The effect of the concentration of hydrogen ions on the kinetics of reactions with participation of hydrogenases is determined by a large number of factors. Protons are the product of the reaction

$$H_2 \rightleftarrows 2H^+ + 2\bar{e}.$$

Consequently a change in their concentration entails a shift of the thermodynamic equilibrium and it is known that a shift of the equilibrium hydrogen potential per pH unit is 59.2 mV. Thus the activity of electron acceptors in the oxidation of H_2 should also depend on the pH. Indeed, the optimum absorption of hydrogen is as a rule attained in the region of alkaline pH, while the optimum evolution occurs in the region of acid pH.[13-15]

However, for certain hydrogenases, for example, those from *Chromatium sp.*,[22] *Th. roseopersicina*,[125] and *Rh. capsulata*,[126] the rate of oxidation of hydrogen by methylviologen in the pH range 7–9 is independent of the proton concentration. The kinetics of the catalysis by hydrogenases will be examined in the next Section.

The proton concentration can influence not only the thermodynamic and kinetic parameters of the reaction itself but also a whole series of other factors. Firstly, one cannot rule out the presence in the protein of ionogenic groups which influence the catalysis. Secondly, the oxidation-reduction transitions in the iron–sulphur clusters and nickel centres also depend on the pH. Thirdly, the proton concentration can influence the properties of the electron transferring and reducing agents. For example, a shift of the pH optimum has been observed[19] in the deuterium exchange reaction catalysed by the hydrogenase from *D. desulfuricans* on activation by various reducing agents.

It is believed that the reduced form of methylviologen is capable of being protonated and decolorised in the range pH < 6.0.[15] Fernandez and co-workers[126] explained the decolorisation of mV^+ in acid solutions, observed by other workers, by the loss of the reducing capacity of sodium dithionite, whose oxidation-reduction potential depends on the pH. In the reduction of methylviologen by metallic zinc, the extinction coefficient of the reduced form of the former was independent of the pH up to 5.0.[127]

V. KINETICS OF THE REACTIONS CATALYSED BY HYDROGENASES

Both in nature and in artificial systems hydrogenases can catalyse reactions of two types. On the one hand, they activate molecular hydrogen for subsequent reactions, including the oxidation of H_2. On the other hand, hydrogenases are capable of catalysing the evolution of hydrogen. The deuterium exchange and hydrogen ortho–para conversion reactions include both processes. Overall, these enzymes accelerate the equilibration in the system

$$H_2 \rightleftarrows 2H^+ + 2\bar{e}.$$

The above reactions can be used to measure the hydrogenase activity. The methods involving the evolution of hydrogen should be especially considered. It has been found[128] that, when the reaction mixture is shaken at a frequency greater than 200 cycles per minute, the H_2 evolution process is not controlled by diffusion. The above conditions are still used in most investigations. However, judging from the latest data,[63] it is impossible to attain the independence of the rate of evolution of hydrogen of the rate of stirring and of the area of the interface between the liquid and gas phases, i.e. the processes associated with the evolution of H_2 are nevertheless complicated by diffusion. The method for the determination of the concentration of hydrogen in solution at the Clark electrode[129] also suffers from a series of disadvantages. Furthermore, the evolution of H_2 from the reduced forms of methylviologen and other electron transferring agents can be complicated by the re-reduction of the hydrogenase. We shall therefore concentrate attention on the kinetics of the processes associated with the activation of hydrogen.

1. Activation of the Hydrogen Molecule at the Active Centres of Hydrogenases

It has been suggested that the rate-limiting stage of the process is the cleavage of the H_2 molecule in the active centres of hydrogenases.[125] However, the order of the reaction involving the oxidation of hydrogen by methylviologen in the presence of hydrogenases from *Th. roseopersicina*[130] and *Rh. capsulata*[126] with respect to H_2 is zero. The study of the isotope effects observed when hydrogen is replaced by deuterium in the activation process with participation of the hydrogenases from *A. eutrophus*, *D. desulfuricans*, and *Rh. japonicum*,[92,131,132] confirmed that the cleavage of the H_2 molecule is the rate-determining stage in the reaction.

The question of the nature of this cleavage is of fundamental importance for catalysis. There exists the view that the cleavage of hydrogen in the active centres of hydrogenases takes place heterolytically. The view is based, in particular, on a study[133] where only the intermediate HD was detected in the gas phase in the course of an investigation of the hydrogenase-catalysed isotope exchange between H_2 and D_2O. However, the final exchange product D_2 was detected in the same system.[128]

Subsequently it was suggested[22] that the rates of formation of the exchange reaction products HD and D_2 be compared. The ratio of the rates v_{HD}/v_{D_2} (v_{HD}/v_{H_2}) depends on a whole series of parameters, for example, on the concentration and activity of the enzyme, so that its numerical values cannot serve as a characteristic of the system. Nevertheless, it is noteworthy that, when the exchange reactions with participation of the hydrogenases from *Chromatium sp.*,[22] *D. desulfuricans*,[134] *D. vulgaris*,[128] *Th. roseopersicina*,[135] *A. eutrophus*,[123] and *Rh. japonicum*[132] were carried out, the rates of formation of HD were of the same order of magnitude as the rates of formation of D_2 and as a rule were not lower than the latter. According to Gitilitz and Krasna,[22] this must be regarded as undoubted evidence in support of the heterolytic mechanism of the cleavage of the H_2 molecule, because at a platinum catalyst the product HD is almost absent, in contrast to D_2. However, platinum is a many-centre catalyst, so that each hydrogen atom behaves independently after the dissociation of the molecule, while the active centre of the hydrogenase is an isolated electronic system with rigorously defined oxidation-reduction electronic transitions.

The ratio of the rates of formation of the intermediate and the exchange products falls with increase of the enzyme concentration, which supports, together with the data quoted above, the model

$$H_2 + E \rightleftharpoons EH^- + H^+.$$

It has been found that the ratio HD/D_2 is independent of the partial pressure of hydrogen.[128] This finding does not disprove the model (2), because it can be accounted for also within the framework of this scheme, for example, when the orders of the formation reactions of HD and D_2 with respect to H_2 are the same.

The dependence of the rate of deuterium exchange in the presence of the hydrogenase from *Rh. japonicum*[132] on the pH parallels the influence of the pH on the rate of evolution of hydrogen. On the other hand, the rate of absorption of H_2 is relatively independent of the proton concentration within the same limits.

The above results support the mechanism of the activation of hydrogen via the intermediate formation of an enzyme–hydride complex EH^-. There are no important facts in the literature in conflict with this mechanism. Furthermore, in a number of studies on the kinetics of reactions with participation of hydrogenases there are direct indications of the possibility that the process occurs via the intermediate formation of EH^-.[52,123,128,132]

However, this does not as yet permit conclusions about the mechanism of the activation and cleavage of the hydrogen molecule in the active centres of hydrogenases. It is appropriate to quote at this point the energy cycle for the reaction between H_2 and protons[136] (the numerical values are given in kcal mol^{-1}):

$$\begin{array}{ccc} H_2 & \xrightarrow{101} & 2H \\ {\scriptstyle 398}\downarrow & & \downarrow{\scriptstyle 2\times 312} \\ H^- + H^+ & \xrightarrow[327]{} & 2H^+ \end{array}.$$

For reactions in an aqueous medium, it is essential to take into account the solvation energy.[137] Thus only 33 kcal mol^{-1} has to be expended on the heterolytic dissociation of the hydrogen molecule in solution.

The active centres of one-centre hydrogenases contain the $[Fe_4S_4]^{2+}$ cluster and Ni^{2+}. Nor can one rule out the possibility of the homolytic dissociation of the H_2 molecule as a result of the redistribution of the electron density and the formation of a covalent bond. Next, as a result of the rapid intramolecular reaction in the active centre of the hydrogenase, there is a possibility of the liberation of protons and of the formation of the EH form in order to compensate for the energy expenditure.

We shall now proceed to the discussion of the activation of the H_2 molecule in the active centres of hydrogenases in the course of its oxidation. We shall consider initially the question of the mutual effect of two substrates: the electron acceptor and H_2. As already stated, carbon monoxide is a competitive inhibitor with respect to hydrogen and a non-competitive inhibitor with respect to the electron acceptor. Study of the mutual effect of H_2 and the oxidised form of methylviologen showed that, in the case of the hydrogenases from *Cl. pasterianum*,[108,138] *M. thermoautotrophicum*,[109] and *Rh. japonicum*,[107] these substrates react with different sections of the active centres. On the other hand, the electron acceptors whose nature is significantly different, namely the high-molecular-weight cytochrome c_3 and the low-molecular-weight methylviologen, interact with the same section of the active centre of the hydrogenase from *D. vulgaris*.[139] Thus the electron acceptor and H_2 molecules each add to their own section of the active centres of the hydrogenases in the hydrogen oxidation reaction.

The processes in which hydrogen is activated by hydrogenases are fairly rapid. In the H_2 oxidation reaction catalysed by the one-centre enzymes from *Th. roseopersicina*[130] and *Rh. capsulata*,[126] the reaction is of zero order with respect to hydrogen. This finding is fairly unexpected for the kinetics of processes with participation of hydrogenases. The dependence on the hydrogen concentration of the oxidation of H_2 by methylviologen had been described earlier for the hydrogenases from *Cl. pasterianum*[108,138] and *D. vulgaris*[140] by the classical Michaelis function. The non-zero reaction order with respect to H_2 is manifested also in the oxidation of the latter by Methylene Blue in the presence of the hydrogenase from *Rh. japonicum*.[107]

The difference between the enzymes from *Th. roseopersicina* and *Rh. capsulata*, on the one hand, and the enzymes from *Cl. pasterianum* and *D. vulgaris*, on the other, consists in the fact that the former are one-centre enzymes, while the latter are many-centre hydrogenases (see Table). The physical properties of the enzyme from *Rh. japonicum* have been less thoroughly investigated, but, it has been shown[61] that it contains 12 iron atoms per protein molecule.

The kinetic parameters of the reactions with participation of the hydrogenases in this group depend in different ways on the pH. In the course of the oxidation of hydrogen by methylviologen with participation of the enzyme from *Cl. pasterianum*,[108] the ratio of the maximum rate of reaction to the Michaelis constant (V_{max}/K_M) increases with decrease of the concentration of protons in the pH range 8–10. V_{max} does not then change and K_M decreases sharply. This confirms indirectly the conclusion that the oxidised form of methylviologen reacts with the enzyme–hydride complex EH^-. Unfortunately, in the remaining cases, it is not possible to discover rigorously the influence of the pH on the reaction kinetics.

In the oxidation of H_2 by methylviologen with participation of the one-centre hydrogenases from *Th. roseopersicina*[125] and *Rh. capsulata*[126] in the pH range 7–9, the maximum rate

and the Michaelis constant are independent of the hydrogen ion concentrations. The parameters of the overall kinetic curves remain unchanged when the pH is varied within the same limits.[130]

The fact that the rate of the enzyme reaction can be of zero order with respect to the substrate and the product is misunderstood in the literature. Thus the authors of a review,[15] criticising the results of Varfolomeev et al.,[125] claim from general considerations that the rate of oxidation of hydrogen "must" depend on the pH.

We shall now consider the observed relations taking into account the known rules governing the occurrence of single-pathway catalytic reactions under stationary conditions (see, for example, Karyakin et al.[141]). The concentration of the product does not enter into the rate equations for such reactions if the product formation stage is irreversible. In order to eliminate the influence of the substrate, a further requirement is that its addition should be much faster than the remaining stages of the reaction. Consequently the zero order of the reaction implies that the addition of the hydrogen molecule and the liberation of protons from the active centres of the one-centre hydrogenases from *Th. roseopersicina* and *Rh. capsulata* should be much faster than the remaining stages, including the oxidation-reduction stages.

A detailed study has been made[130] of the kinetics of the oxidation of hydrogen by methylviologen catalysed by the hydrogenase from *Th. roseopersicina*. In order to discover the sequence of the stages, the dependence of $1/V_{max}$ and K_M/V_{max} for both substrates on the pH and the concentration of the second substrate was determined. Kinetic analysis makes it possible to select the unique sequence

$$H_2 \cdot H^+ — MV^{2+} \rightarrow MV^{2+} — H^+ \rightarrow,$$

where the arrow indicates irreversible stages. Thus, according to Karyakin et al.,[130] the electron acceptor reacts with the enzyme–hydride complex EH^-; after the elimination of the second acceptor molecule from the active centre, the enzyme remains in the protonated form.

Thus kinetic data confirm the conclusion that the activation of the hydrogen molecule in the active centres of hydrogenases leads to the formation of the enzyme–hydride complex.

The kinetic differences between the many-centre and one-centre hydrogenases can be traced in relation to the enzymes considered. While the kinetic parameters of the hydrogen oxidation reactions in the presence of one-centre hydrogenases are independent of the pH and the H_2 concentration, in the presence of many-centre enzymes the reaction can be of zero order with respect to hydrogen and protons. If the rates of the oxidation-reduction stages were comparable for both groups of enzymes, it would follow that the cleavage of the H_2 molecule in the presence of many-centre hydrogenases should be slower than in the presence of the one-centre enzymes. However, the opposite behaviour is observed experimentally: the activity of the many-centre hydrogenases is on average higher by two orders of magnitude than that of the one-centre hydrogenases. Thus the rates of the oxidation-reduction stages are much higher for the many-centre hydrogenases than for the one-centre enzymes. This in fact constitutes the main kinetic difference between them.

It would be of interest to compare also the rates of formation of the enzyme–hydride complex for both groups of hydrogenases. However, this would require the knowledge of the dependence of the Michaelis constant (with respect to hydrogen) on the pH. Unfortunately there are no such data in the literature. The hydrogenase from *Th. roseopersicina*[130] forms EH^- at a rate characterised by a constant of the order of 10^5 min^{-1}, which is comparable to the rate constants for reactions catalysed by many-centre hydrogenases (they are calculated from the maximum rates[13,14] see Table), which are of the order of 10^4–10^5 min^{-1}. Thus the hydrogen molecule is activated with formation of the enzyme–hydride complex equally fast by both groups of hydrogenases. Since the iron–sulphur clusters of the one-centre and many-centre enzymes have extremely different properties, presumably the hydrogen molecule is not activated at this cluster. This conclusion agrees well with the hypothesis that the nickel centre is the acceptor of the H_2 molecule.

The extremely high rate of formation of the enzyme–hydride complex EH^- in the active centres of hydrogenases is noteworthy. The heterolytic dissociation of the H_2 molecule under the conditions of the metal complex catalysis[142] is 10^5–10^6 times slower. However, it is as yet not clear what particular mechanism is responsible for such high rates of the enzyme reactions compared with the reactions in the presence of the familiar model catalysts.

2. The Oxidation-Reduction Stages

We shall consider the interaction of the enzyme with the electron acceptor in the hydrogen oxidation reaction. Firstly, one should note that a dependence of the catalytic properties of enzymes on numerous factors is characteristic of all enzymes. Thus it has been found that cytochrome c_3 appreciably stimulates the activity of the hydrogenase from *D. desulfuricans*.[131] Furthermore, the rate of evolution of hydrogen from the reduced mixture of methylviologen and cytochrome c_3 under the influence of this hydrogenase exceeds the sum of the rates of its evolution from methylviologen and the cytochrome taken separately.[139] Inhibitor analysis shows that both acceptors interact with the same section of the active centre in the enzyme.

Secondly, it has been stated by some investigators[108,123] that the deuterium exchange reaction in the active centres of hydrogenases can include oxidation-reduction stages due to the enzyme–enzyme interaction. The addition of an electron acceptor leads to the inhibition of the deuterium exchange with participation of the hydrogenases from *Cl. pasterianum*,[108] *A. eutrophus*,[123] *and Rh. japonicum*.[132] After the complete reduction of the acceptor, the deuterium exchange is renewed. Consequently the pathway associated with the reduction of the electron transferring agent is tentative.

Finally, as established above, the oxidation-reduction stages are rate-limiting in the catalysis of the oxidation of molecular hydrogen by hydrogenases. The dependence of the rate of oxidation of molecular hydrogen catalysed by hydrogenases on the concentration of a one-electron acceptor (methylviologen, benzylviologen) is as a rule described by the Michaelis function. However, in the catalysis by the hydrogenases from *M. elsdenii*[71,76] and *D. vulgaris*[53] the dependence of the reciprocal of the rate of evolution of hydrogen on the reciprocal of the methylviologen concentration is non-linear. This can be accounted for by side effects, for example, by the formation of the dimer of the reduced form of methylviologen,[143] and by the diffusion or re-reduction of the enzyme, because the non-linear dependence of the rate of evolution of hydrogen in terms of the variables of the Lineweaver–Burk equation was found[53,76] to be coupled with a linear dependence of the absorption of hydrogen. Furthermore, it has been discovered[71] that the H_2 evolution reaction is of first order with respect to methylviologen for the same hydrogenase from *D. vulgaris* (Hildenborough strain) (cf. Grande et al.[53]).

On the other hand, studies[59,139,140] using the hydrogenase from *D. vulgaris* (Miyazaki strain) led to the discovery of a quadratic dependence of the rates of absorption and evolution of hydrogen on the methylviologen concentration. This contradiction was explained somewhat later.

With rare exceptions, the hydrogen oxidation (and formation) reactions are usually found to be of first order with respect to the electron acceptor. Methylviologen and benzylviologen are one-electron mediators and their interaction with the active centre of the enzyme during the reaction should be separated by an irreversible stage.[130,141]

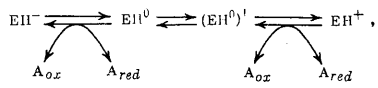
A detailed study has been made[126,130] of the kinetics of the interaction of the enzyme–hydride complex with the electron acceptor. The rate of the hydrogen oxidation reaction is related to the electron acceptor concentration by an expression of the type of the Michaelis equation and the form of the integral curve depends on the concentration of the reduced form of the acceptor. The apparent Michaelis constant is proportional to the concentration of the reduced form of the electron acceptor. Thus the rate equation for the process is

$$v = \frac{V_{max}[S]}{K_M + [S] + K'_p[P]}, \qquad (2)$$

where [S] and [P] are respectively the concentrations of the substrate and the product and K'_p is the product binding constant.

Various possible mechanisms, including the mechanism involving a reversible catalytic stage, the general mechanism with inhibition by the product, and the mechanism with a reversible bimolecular interaction with the substrate have been considered in the course of kinetic analysis. The dependence of the kinetic parameters of Eqn.(3) on the oxidation-reduction potential of the electron acceptor made it possible to give preference to the mechanism with reversible bimolecular interaction:

Scheme 1



where A_{ox} and A_{red} are respectively the oxidised and reduced forms of methylviologen or benzylviologen. Thus a slow intramolecular stage has been discovered in the mechanism of the catalytic reaction.

On the other hand, the H_2 oxidation reaction catalysed by the hydrogenase from *Rh. japonicum* is not inhibited by the reduced form of the electron acceptor [Methylene Blue (MB), phenazine metasulphate (PMS)].[107] The oxidation-reduction potentials of the above acceptors are respectively +11 and +80 mV and differ markedly from the potentials of methylviologen and benzylviologen (−440 and −359 mV respectively). Since the "inhibiting" effect is shown to be associated with the reverse reaction, i.e. with the donation of an electron, the behaviour of reduced MB and PMS becomes entirely understandable. Furthermore, Scheme 1 has been derived for a one-electron transferring agent.

In Scheme 1 there is a unimolecular stage between the stages involving interaction with electron acceptors and it is rate-limiting. Its physical significance becomes understandable if account is taken of the fact that the abstraction of the first electron from the hydride ion in the gas phase required approximately twenty times less energy than the abstraction of the second electron. The ΔH for these processes are respectively 15 and 312 kcal mol^{-1}.[136]

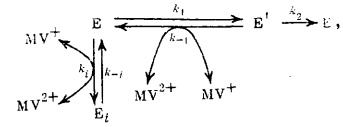
The difference between the many-centre and one-centre hydrogenases may consist both in the different degrees of stabilisation of the intermediate forms and hence in the different ratio of the rates in stages (*1*) and (*3*) in the forward and reverse directions (Scheme 1) and in the acceleration of the intramolecular stage (*2*). As a consequence of this, when the most active hydrogenases are employed, the second order of the reaction with respect to the electron acceptor may be observed. The hydrogenase from *D. vulgaris* (see Table) has established a kind of record as regards activity and the second order of the hydrogen absorption and evolution reactions with respect to methylviologen, observed in the presence of this enzyme, is quite readily explicable.

However, it has been shown[126] that the second order of the reaction with respect to methylviologen is also observed in the hydrogen oxidation reaction in the presence of the hydrogenase from *Rh. capsulata*. This enzyme is a striking representative of the group of one-centre hydrogenases (see Table) and there is therefore no analogy with the hydrogenase from *D. vulgaris*. We may recall that the enzyme from *Rh. capsulata* belongs to the group of "one-centre" hydrogenases:[60,75] The question arises what can be the characteristic features of the enzymes of this group.

A catalyst is incapable of shifting the thermodynamic equilibrium of the reaction. Consequently the only possible explanation is the creation of kinetic hindrance: the acceleration of the forward reaction of the catalyst and its "switching off" along the reverse pathway. It has been suggested[63] that the inactivating re-reduction of one-sided hydrogenases occurs even in the range of positive potentials relative to the hydrogen electrode in the same solution. Thus, under conditions which are thermodynamically favourable for the evolution of hydrogen, the hydrogenase becomes inactive. The inactivating re-reduction potential for the enzyme from *Rh. capsulata*[75] proved to be −340 mV at pH 7.0. The change in potential per pH unit is then approximately −30 mV.

Only the reduced electron acceptor can serve as the electron donor for the re-reduction in the hydrogen oxidation reaction. The simplest scheme of the oxidation-reduction stages in the catalysis by the hydrogenase by *Rh. capsulata* is as follows:

Scheme 2



where E_i is the inactive form of the catalyst.

Using the corresponding rate equation, it is possible to calculate from kinetic data the inactivating re-reduction potential ($E_i \cong -387 \pm 5$ mV at pH 8.5). This value agrees well with that found by oxidation-reduction titration ($E_i \cong -385 \pm 5$ mV).[75] Thus the hypothesis of the inactivating re-reduction as the basis for the regulation of "one-sided" hydrogenases has been confirmed in a direct kinetic study. It must be emphasised that the conclusion that common mechanisms of the catalysis operate has been extended also to the group of "one-sided" enzymes. Their only difference from the two-sided hydrogenases from the kinetic standpoint is the presence of the characteristic inactivating re-reduction stage.

In conclusion we present the final scheme for the catalysis by the hydrogenase from *Th. roseopersicina* (Scheme 2 based on the data of Karyakin and co-workers[126,130]), which is fairly general for hydrogenases from different

sources:

Scheme 3

$$E + H_2 \overset{K_1}{\rightleftarrows} EH_2, \quad (1)$$

$$EH_2 \overset{K_2}{\rightleftarrows} EH^- + H^+, \quad (2)$$

$$EH^- + A_{ox} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} EH^0 + A_{red}, \quad (3)$$

$$EH^0 \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} (EH^0)', \quad (4)$$

$$(EH^0)' + A_{ox} \underset{k_{-5}}{\overset{k_5}{\rightleftarrows}} EH^+ + A_{red}, \quad (5)$$

$$EH^+ \overset{}{\rightleftarrows} E + H^+, \quad (6)$$

where E is the hydrogenase and A is methylviologen or benzylviologen. Stages (*1*), (*2*), and (*6*) are of the quasi-equilibrium type. Here the equilibrium is strongly displaced towards the formation of products, so that the occurrence of any kind of irreversibility in the above set of stages is entirely well founded. Stage (*4*) is also irreversible within the framework of the given kinetic conditions. Since the adjoining stages (*3*) and (*5*) can proceed in both forward and reverse directions while stage (*4*) is the slowest, it is reasonable to postulate the simplest type of irreversibility: $[A_{ox}]k_5 \gg k_{-4}$. We may note that the restrictions on the reaction conditions thus obtained involve the concentrations of the reactants. Thus, by changing the process conditions, it is possible to achieve not only reversibility along the sections indicated but also irreversibility in the opposite direction.

VI. KINETICS OF THE ELECTROCHEMICAL PROCESSES CATALYSED BY HYDROGENASES

The phenomenon of the acceleration of electrode processes by enzymes has been called "bioelectrocatalysis".[7-11] In this case the electrode behaves as one of the reaction substrates, the electron donor, or the electron acceptor. There are two ways of coupling enzyme and electrochemical reactions: the mediator mechanism in which the electron exchange is effected by a transferring agent, capable of reacting both with the electrode and with the active centre of the enzyme, and the direct mechanism in which the enzyme is capable of being directly oxidised or reduced at the electrode.

The mediator bioelectrocatalysis has been achieved using the hydrogenases from *Pr. aestuarii* and *Th. roseopersicina*.[144] It has been shown that the conditions in the oxidation of hydrogen are close to reversibility: depending on the electrode potential, such a system can operate both with absorption and in evolution of hydrogen.

A unique feature of the hydrogenase from *Th. roseopersicina* is its ability to behave as the electrocatalyst of the oxidation-formation of molecular hydrogen in sorption on the surface of a carbon black electrode.[145] It has been shown that, in a H_2 atmosphere on an electrode with the immobilised hydrogenase, an equilibrium hydrogen potential is established. In the hydrogen oxidation reaction the current drawn from the electrode is independent of the pH and the partial H_2 pressure over the entire overpotential range.[126] The zero order of the electrocatalytic reaction with respect to the substrate and the product permits the conclusion, as in homogeneous reactions, that the addition of a hydrogen molecule and the liberation of protons from the active centre of the enzyme are rapid compared with the oxidation-reduction (in the given instance electrochemical) stages.

The overall polarisation curve for the electrochemical oxidation of H_2 is described by the equation corresponding to the occurrence of two consecutive potential-dependent stages involving electron transfer from the active centre of the enzyme to the electrode.[126] It has been shown that the electrochemical stages must follow one after another, i.e. cannot be separated by addition–dissociation processes. The mechanism of the homogeneous reaction includes, in addition, an intramolecular stage separating two oxidation-reduction stages. Evidently in electrocatalysis this stage is concealed in the electrochemical stages; one can only observe that the second electron is transferred to the electrode more slowly than the first.

The parameters of the positive branch of the polarisation curve make it possible to predict the kinetics of the reverse reaction, i.e. of the electroevolution of hydrogen. The negative branch has inferior current characteristics compared with the positive component. Thus the use of the hydrogenase electrode is most justified in the H_2 oxidation reaction. Furthermore, the inactivating re-reduction of the active centre of the hydrogenase has been detected in the electrochemical system, which constitutes evidence for the occurrence of this phenomenon, since the possibility of the effect by any other inactivating factors has been ruled out experimentally.

Thus the study of the kinetics of the electrochemical reaction with participation of the hydrogenase from *Th. roseopersicina* not only confirms but also supplements the conclusions concerning the characteristic features of the homogeneous kinetics and the catalytic properties of hydrogenases reached in the previous Section.

VII. CONCLUSIONS

This review has dealt with the most general physicochemical properties of hydrogenases, the structure of their active centres, and the characteristic features of the catalysis. In conclusion we should like to put forward the hypothesis that the action of these enzymes has a molecular mechanism.

We have been able to divide hydrogenases into two groups differing in their physical properties, activities, and kinetic parameters in the hydrogen oxidation reaction. The one-centre hydrogenases contain one 4Fe–4S cluster. Their activity is on average lower by two orders of magnitude than that of the many-centre hydrogenases containing several iron–sulphur clusters in the active centre. While the addition of a hydrogen molecule and the liberation of protons are effected equally rapidly by both groups of hydrogenases, an appreciable difference is observed in the rates of the oxidation-reduction stages. The latter evidently proceed with participation of the iron–sulphur structures in the active centre.

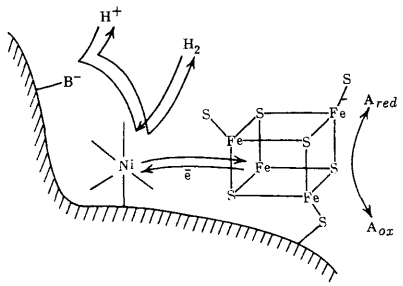
Furthermore, it has been possible to elucidate the kinetic scheme for the hydrogenase catalysis. The activation of the hydrogen molecule in the active centres of hydrogenases leads to the formation of the enzyme–hydride complex EH^-. In the H_2 oxidation reaction the interaction of the one-electron acceptor with the active centre is a reversible bimolecular process. The addition of the second acceptor molecule is preceded by a slow intramolecular stage.

The involvement of nickel in hydrogenase catalysis is indisputable. This has been confirmed, firstly, by the overlap of the electron orbitals of the nickel and iron–sulphur centres. Secondly, inhibitor analysis and the character of the mutual influence of substrates indicate that the H_2 and electron acceptor molecules interact with different sections of the active centre. Furthermore, a parallel has been

traced between the relative equality of the rates of activation of the hydrogen molecule by the one-centre and many-centre hydrogenases and the identity of the oxidation-reduction potentials of their nickel centres, while the properties of the iron–sulphur cluster structures in these two types of hydrogenases differ significantly. Finally, the one-electron reduction of Ni^{3+} to Ni^{2+} requires the participation of a proton acceptor. All these factors support the view that the nickel centre is the initial acceptor of the H_2 molecule.

The molecular mechanism of the catalysis by hydrogenases of the hydrogen oxidation reaction can be described with the aid of a two-centre model:

Scheme 3



The H_2 molecule is sorbed on a "free" coordination site of nickel, substituting, for example, a water molecule. This is followed by heterolytic dissociation with formation of the enzyme–hydride complex apparently without the involvement of the electron density of the iron–sulphur cluster. The latter is also able to accept one of the electrons of the hydride rapidly and reversibly. The iron–sulphur cluster interacts with the acceptor, giving up the "superfluous" electron, also rapidly and reversibly. The abstraction of the second electron from the hydride ion is known to require the expenditure of more energy by 300 kcal mol^{-1} than the abstraction of the first electron. Consequently, the transfer of the second electron between the nickel and iron–sulphur centres is much slower. This stage is in fact manifested in the kinetics as the slow intramolecular stage. It is also followed by the rapid interaction with the acceptor and by deprotonation. As a result of the superior organisation of the iron–sulphur clusters, many-centre hydrogenases are capable of effecting the intramolecular transfer of the second electron at a higher rate. Overall, the role of the iron–sulphur clusters in hydrogenases consists in the stabilisation of the intermediate electronic states, as in other enzymes, for example, in nitrogenase. The presence of a spatially branched structure is essential for the abolition of the change in the ambient medium when the oxidation state is altered, which leads to a decrease of the activation energy for the electron transfer according to the solvent reorganisation theory.

In the oxidised state, hydrogenases are inactive in the hydrogen oxidation reaction and the possibility of the activation of the H_2 molecule is lost. It would appear that the electric field of Ni^{3+} should promote the heterolytic dissociation of the H_2 molecule to a greater extent than the Ni^{2+} field. The first step in the activation of the H_2 molecule apparently consists in the transfer of an electron to the latter from the active centre of the hydrogenase, which appreciably weakens the H–H bond. The potential of the $Ni^{3+} \leftrightarrow Ni^{2+}$ transition (−150 mV) allows fully the possibility of such donation. If this were followed by the reduction of Ni^{3+} to Ni^{2+}, for example, as a result of the reduction of the iron–sulphur cluster $[Fe_4S_4]^{2+}$, then a proton-acceptor group would appear in the coordination sphere of Ni^{2+}. Thus the following conditions for the dissociation of the H_2 molecule arise in the transition state obtained: (1) the weakening of the H–H bond as a result of the appearance of an electron in the antibonding orbital of the molecule; (2) the presence of a bivalent nickel atom capable of accepting a hydride ion; (3) the appearance of a proton acceptor in the coordination sphere of the nickel centre; (4) the formation of an oxidised iron–sulphur cluster capable of accepting an electron.

As already mentioned, hydrogenases activate the hydrogen molecule and effect the formation of the enzyme–hydride complex at extremely high rates compared with metal complex catalysts. The hydrogenase from *A.eutrophus* is capable of hydrogenating NAD to NADH. Thus specific modification of the active centres of hydrogenases or their modelling leads to a way of creating more effective catalysts of the hydrogenation and dehydrogenation of organic compounds.

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