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# Demonstration of two different $H_2$-oxidizing activities in soil using an $H_2$ consumption and a tritium exchange assay

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**Abstract** $H_2$-oxidizing activities were assayed in slurries of four soils by measuring the consumption of $H_2$ and the exchange of $^3H_2$ with $H_2O$ at increasing mixing ratios of $H_2$ or $^3H_2$. Both $H_2$ consumption and $^3H_2$ exchange were abolished by autoclaving or the addition of formaldehyde. The rates of $H_2$ consumption and $^3H_2$ exchange were proportional to the quantity of soil used. Both activities increased with increasing concentrations of $H_2$ or $^3H_2$ and displayed biphasic kinetics, demonstrating the existence of two different $H_2$-oxidizing activities, one with a relatively low $K_m$ and $V_{max}$, and a second with a relatively high $K_m$ und $V_{max}$. The first type of activity was characteristic of abiontic soil hydrogenases, and the second of aerobic $H_2$-oxidizing bacteria. In contrast to $H_2$ consumption, which required the presence of either $O_2$ or ferricyanide, $^3H_2$ exchange operated equally well without an external electron acceptor. The $^3H_2$ exchange assay may thus be particularly useful for enrichment of soil hydrogenases which have not yet been isolated and for which no natural electron acceptor is known.

**Key words** Soil hydrogenase · Knallgas bacteria · Tritium exchange · Hydrogen consumption · $V_{max}$ · $K_m$ · Electron acceptor

## Introduction

Molecular $H_2$ is an atmospheric trace gas which is produced by various biogenic and abiogenic processes (Seiler 1978; Conrad 1988). Due to anthropogenic emissions the abundance of $H_2$ seems to be increasing by about 0.6% per year, and may add more water vapour to the stratosphere and thus affect the ozone layer (Khalil and Rasmussen 1990). Atmospheric $H_2$ is almost exclusively decomposed in soil by oxidation processes which are not well characterized (Conrad 1988). Indirect evidence suggests that atmospheric $H_2$ concentrations (0.55 ppmv) are oxidized by abiontic soil hydrogenases (Conrad and Seiler 1981; Conrad et al. 1983). However, these soil hydrogenases have not been isolated and characterized. Recent studies have shown that two different $H_2$-oxidation activities can be distinguished in soil on the basis of their $K_m$ values (Schuler and Conrad 1990). Activities with a low and a high $K_m$ were attributed to soil hydrogenases (high-affinity activity) and to aerobic $H_2$-oxidizing bacteria (low-affinity activity), respectively.

A problem in isolating soil hydrogenases is the lack of a convenient assay, since the natural electron acceptor of the enzyme is unknown but must be present for enzyme activity to take place. This problem may be overcome by using the tritium ($^3H_2$) exchange reaction as an assay for hydrogenases. This reaction is independent of external electron acceptors and allows measurement of the activity of hydrogenases (Schink et al. 1983).

Conversion of $^3H_2$ to $^3H_2O$ and $^3HHO$ has been demonstrated in soils (McFarlane et al. 1978; Fallon 1982; Förstel 1986). The properties of this conversion reaction indicated that it was catalysed by soil hydrogenases. However, no direct comparison has been made between $H_2$ consumption and $^3H_2$ exchange. Therefore, we studied the kinetics of $H_2$ consumption and of $^3H_2$ exchange in different soil types.

## Materials and methods

Samples were taken from the upper 10 cm of the soil profile after removal of litter. One soil was from the ground of a horse-riding hall. The other soils were taken from sites which have already been described, a cambisol from the Bavarian Forest (Saad and Conrad 1993), a calcic cambisol from a meadow, and a luvisol from a forest near Konstanz (Baumgärtner and Conrad 1992). The field-moist soil samples were passed through a screen (2 mm mesh) and were stored in polyethylene bottles at 1 °C. Soil texture, organic C, and total N were analysed by a soil analytical laboratory (Standort, Stuttgart, Germany) using standard protocols (Schlichting und Blume 1966). The characteristics of the soils are given in Table 1.

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**Table 1** Characteristics of the soils. Origin of Soils: BF, Bavarian Forest; KBE, meadow near Konstanz; PBE, forest near Konstanz; RH, ground of a horse-riding hall. WHC, Water-holding capacity

| | Soil | | | |
|---|---|---|---|---|
| | BF | KBE | PBE | RH |
| Type | Clay silt Cambisol | Sandy silty loam Calcic cambisol | Silty loam Luvisol | |
| Texture (%) | | | | |
| Clay (<2 µm) | 9 | 30 | 15 | |
| Silt (2–63 µm) | 61 | 47 | 63 | |
| Sand (63–2000 µm) | 30 | 23 | 22 | |
| pH ($H_2O$) | 4.5 | 7.8 | 5.0 | 7.1 |
| Organic C (%) | 13.5 | 4.6 | 3.3 | |
| Total N (%) | 1.2 | 0.42 | 0.1 | |
| C:N | 11.3 | 9.8 | 33.0 | |
| WHC (g $g^{-1}$ dry weight) | 1.80 | 0.77 | 0.70 | 3.7 |

The rates of $H_2$ consumption were measured by placing 40 mg fresh soil into serum bottles (120 ml) containing 10 ml sterile water. The bottles were flushed with $H_2$-free synthetic air (80% $N_2$, 20% $O_2$), closed with black rubber stoppers, and pressurized to 1.2 bar. The desired quantity of $H_2$ was injected and the bottles were incubated at 20 °C on a shaker (150 rpm). The decrease in $H_2$ was followed over time by repeatedly taking gas samples (2 ml) with gastight syringes and analysing the $H_2$ mixing ratio by gas chromatography (Schuler and Conrad 1990). We ensured that the rate of $H_2$ consumption remained proportional to the mass of soil in order to exclude any limitation of $H_2$ consumption by the transfer of $H_2$ from the gas phase into the aqueous phase. Proportionality was present for up to 50 mg field-moist soil. $H_2$ consumption rates were measured at different initial $H_2$ mixing ratios and were expressed on a gram dry weight basis. The $H_2$ consumption rates were plotted as a function of the initial $H_2$ concentrations using the Michaelis-Menten equation in the form of the Eadie-Hofstee plot to determine $V_{max}$ and $K_m$.

The rates of $^3H_2$ exchange were determined using a similar method as that used for measuring $H_2$ consumption with $^3H_2$ instead of $H_2$ injected into the bottles. Gaseous $^3H_2$ was prepared from $^3H_2O$ (8.9 Ci $mol^{-1}$; Amersham-Buchler, Braunschweig) by reaction with metallic lithium ($2\,H_2O + 2\,Li = 2\,LiOH + H_2$; Hallahan et al. 1986). The time-course of the exchange reaction was followed by repeatedly taking liquid samples (0.6 ml) from the suspension with a syringe, separating the aqueous phase from the solid phase by centrifugation (1000 rpm), mixing 0.4 ml of the supernatant with 4 ml scintillation cocktail (Quicksafe A, Zinsser, Frankfurt), and measuring the radioactivity in a scintillation counter (Packard Tri-Carb 1990 A). Quenching effects were corrected by external standardization. Occasionally, quench correction was checked by internal standardization using $^3H_2O$. Again, we ensured that the rate of $^3H_2$ exchange, i.e. the incorporation of radioactivity into $H_2O$, was porportional to the mass of soil. When all of the $^3H_2$ was consumed, the total recovery of radioactivity in $H_2O$ and soil was 70%. Inhibition of the $^3H_2$ exchange reaction was tested under a headspace of 100% CO, 100% acetylene, or after the addition of formaldehyde (3.4% final concentration).

In some experiments, soil was incubated under anoxic conditions, by suspending the soil in degassed sterile water and flushing the bottles with $N_2$. The suspension was pre-incubated overnight (about 15 h) in presence of 10 ppmv $H_2$ and then evacuated and flushed with $N_2$. Ferricyanide {$K_3$ [$Fe(CN)_6$]} was added as an external electron acceptor to a final concentration of 5 m*M*.

## Results

The determination of $H_2$ oxidation rates as a function of increasing concentrations of $H_2$ showed biphasic kinetics, both when assayed by $H_2$ consumption (Fig. 1 A) and by $^3H_2$ exchange (Fig. 1 B). The kinetics allowed a dis-

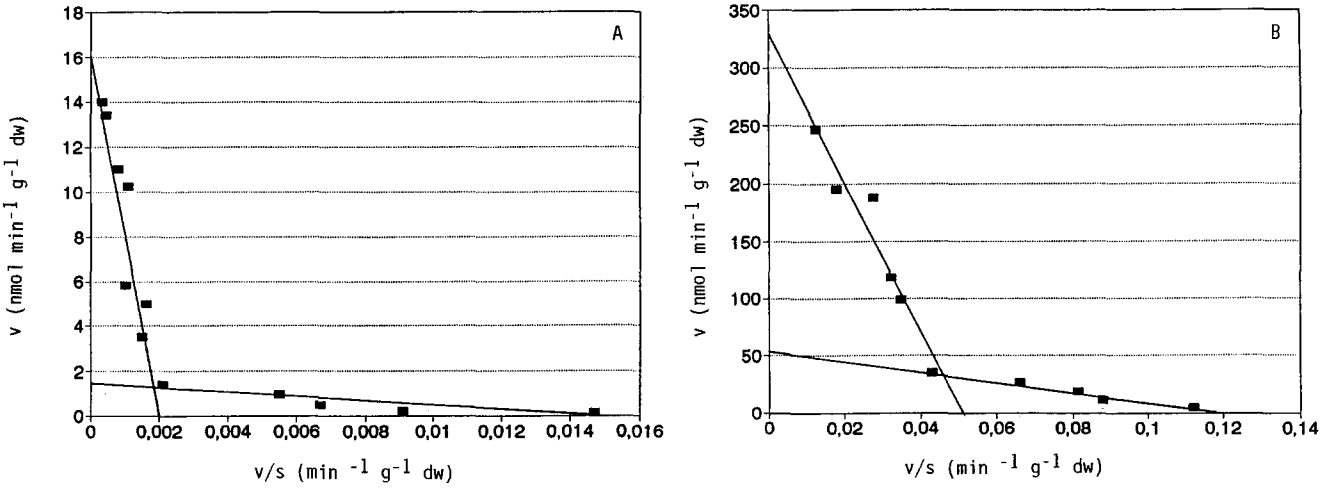


**Fig. 1 A, B** Eadie-Hofstee plot describing the kinetics of $H_2$ oxidation in soil slurries: **A** kinetics of $H_2$ consumption in soil from a meadow near Konstanz; **B** kinetics of $^3H_2$ exchange in a Bavarian Forest soil. *dw*, Dry weight

**Table 2** Kinetic parameters of $H_2$ consumption and $^3H_2$ exchange in different soils, determined from Eadie-Hofstee plots. For origin of soils, see Table 1

| Soil | Assay | Activity | $V_{max}$ (nmol $min^{-1}$ $g^{-1}$ dry weight) | $K_m$ | |
|---|---|---|---|---|---|
| | | | | ppmv | nM |
| BF | $H_2$ | 1 | 7.9 | 27.5 | 21.1 |
| | | 2 | 134 | 1040 | 780 |
| | $^3H_2$ | 1 | 56.1 | 101 | 82.3 |
| | | 2 | 333 | 1450 | 1080 |
| KBE | $H_2$ | 1 | 1.4 | 19.1 | 12.3 |
| | | 2 | 17.0 | 1430 | 1075 |
| | $^3H_2$ | 1 | 36.1 | 123 | 93.5 |
| | | 2 | 209 | 1240 | 910 |
| PBE | $H_2$ | 1 | 0.6 | 19.8 | 13.2 |
| | | 2 | 77.4 | 1520 | 1100 |
| | $^3H_2$ | 1 | 15.4 | 78.3 | 64.2 |
| | | 2 | 83.5 | 1120 | 805 |
| RH | $H_2$ | 1 | 9.9 | 91.4 | 73.2 |
| | | 2 | 33.1 | 800 | 621 |
| | $^3H_2$ | 1 | 91.6 | 74.5 | 63.2 |
| | | 2 | 727 | 1470 | 1080 |

tinction between two different activities. The $V_{max}$ and $K_m$ which were determined for these two activities in four different soils are summarized in Table 2. In general, the first type of activity (high-affinity activity), which was mainly operating at low $H_2$ mixing ratios, showed $K_m$ values $< 100$ n$M$ $H_2$ and also showed relatively low $V_{max}$ values. In contrast, the second activity (low-affinity activity), which was mainly operating at higher $H_2$ mixing ratios, showed $K_m$ values of $> 1000$ n$M$ $H_2$ and relatively high $V_{max}$ values.

The $V_{max}$ values determined by the $^3H_2$ exchange reaction were generally higher than those determined by the $H_2$ consumption assay. The $K_m$ values of the high-affinity activity in three out of four soils were also higher when determined by $^3H_2$ exchange than by $H_2$ consumption. However, they were still much lower than those of the low-affinity activity.

Consumption of $H_2$ by soil did not occur when the soil was incubated in the absence of $O_2$. $H_2$ consumption did take place, however, when $O_2$ was replaced by ferricyanide as an alternative electron acceptor (Table 3). The $^3H_2$ exchange reaction, in contrast, was independent of the presence of an external electron acceptor and took place equally well under oxic and anoxic conditions and in the presence or absence of ferricyanide (Table 3).

The activity of the $^3H_2$ exchange reaction was completely eliminated by autoclaving the soil or by treatment with formaldehyde. However, the $^3H_2$ exchange reaction was relatively insensitive to acetylene (6–19% reduction) or CO (17–64% reduction).

**Table 3** Effect of ferricyanide as an exogenous electron acceptor on the activity of $H_2$ oxidation and $^3H_2$ exchange under anoxic conditions at a low (1 ppmv) $H_2$ mixing ratio. For origin of soils, see Table 1

| Soil | Assay | Anoxic activity (% of oxic control) | |
|---|---|---|---|
| | | No acceptor | $K_3[Fe(CN)_6]$ |
| BF | $H_2$ | 0 | 98 |
| | $^3H_2$ | 101 | 102 |
| KBE | $H_2$ | 0 | 97 |
| | $^3H_2$ | 99 | 102 |

## Discussion

Our results confirm and extend earlier observations concerning the existence of two different $H_2$-oxidizing activities in soils (Schuler and Conrad 1990). The presence of these activities was shown by using various soils and two different assays. In contrast to the studies of Schuler and Conrad (1990), who tested the $H_2$-oxidizing activities in soil samples at quasi-natural moisture contents, the present study applied the assays to soil suspensions, which are easier to handle. All the assays resulted in the detection of a low- and a high-affinity activity for $H_2$ oxidation.

One of the assays involved measurement of the net consumption of $H_2$ catalysed by $H_2$-oxidizing activities in the soil. This assay required the presence of a suitable electron acceptor, i.e., either $O_2$ or ferricyanide. Ferricyanide and other electron acceptors with redox potentials $> 80$ mV can replace $O_2$ in anoxic assays (Conrad et al. 1983). The other assay used in the present study measured the exchange of $^3H_2$ with $H_2O$. This reaction is catalysed by the reaction center of the hydrogenase and thus does not require the presence of an external electron acceptor. Because of the irrelevance of the electron acceptor, the $^3H_2$ exchange reaction is a useful assay for attempting enrichment and isolation of the still uncharacterized soil hydrogenases which are presumably responsible for the high-affinity activity. Using this assay avoids the need to know the natural electron acceptor of this soil enzyme. The $^3H_2$ exchange reaction has also been used to measure hydrogenase activities in aquatic environments (Paerl 1983; Schink et al. 1983).

Both CO and acetylene are commonly used to inhibit hydrogenase activity, although not every hydrogenase is inhibited, (Adams et al. 1981; Oremland and Capone 1988). In the two soils investigated, the inhibitory effect of acetylene was small (6–19%), and CO also did not result in complete inhibition (17–64%), even though both inhibitors were applied at the maximum concentration (i.e., 100% gas phase). In general, the high-affinity activity seems to be relatively insensitive to inhibitors (Conrad and Seiler 1980). However, there is no doubt that the activity was biotic because it was completely destroyed by autoclaving or by treatment with formaldehyde. Furthermore, it exhibits a temperature optimum at about 35–40 °C (Schuler and Conrad 1991). The high resistance of the high-affinity activity to inhibitors, desiccation, and antibiotics (Conrad and Seiler 1981) is characteristic of

an abiontic soil enzyme as defined by Skujins (1978). The importance of the activity in the global cycling of atmospheric $H_2$ (Conrad 1988) indicates the need to isolated and characterize soil hydrogenases.

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