

Sulfide Stimulation of Light-Induced Hydrogen Evolution by the Cyanobacterium *Nostoc muscorum*

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Light-induced hydrogen evolution by the blue-green alga *Nostoc muscorum* is stimulated by sulfide with a rate of about 20 $\mu\text{mol H}_2$ per mg chlorophyll and hour stable for about one day at low light intensities (7 W/m²). Oxygen evolution does not impair sulfide-stimulated hydrogen formation. Increased hydrogen evolution is catalyzed by nitrogenase, its activity is not influenced by sulfide. Apparently, sulfide inhibits the hydrogen-uptake pathway(s), thereby increasing net hydrogen evolution. – Furthermore, photosynthetic electron flow is inhibited by sulfide, essentially at the oxidizing side of photosystem II, as was demonstrated by fluorescence-signal changes. This leads to decreased electron supply of nitrogenase.

Hydrogen production apparently is at optimum when both the effect of sulfide on photosynthesis is moderate and when uptake-nitrogenase activity is abolished.

Introduction

Besides dinitrogen reduction, hydrogen evolution by heterocystous cyanobacteria is catalyzed by nitrogenase [1, 2]. It is our goal to increase the latter activity at the expense of the first one. As has been shown previously, hydrogen evolution is increased by preincubation of blue-greens like *Nostoc muscorum* under resting conditions, *i. e.* in the absence of dinitrogen and CO₂ [3] or by preincubation with low concentrations of acetylene [4]. Photosynthetic electron-transport inhibitors are also useful to increase the light-induced hydrogen evolution by heterocystous cyanobacteria, when grown under certain physiological conditions [3, 5]. Incubation with sulfide, to be reported herein, appears to be an additional means to substantially stimulate light-induced net hydrogen evolution.

Oscillatoria limnetica is able to oxidize sulfide and evolve hydrogen via photosystem I and hydrogenase [6]. Also oxidation of sulfide under anaerobic conditions take place in certain photosynthetic bacteria and cyanobacteria [7]. Anoxigenic, bacterial-like photosynthesis with sulfide oxidation is equally found in some non-heterocystous cyanobacteria [8]. Further, sulfide inhibits photosynthetic electron transport between water and photosystem II

[9]. These effects had to be taken into account during this study.

Data are presented to describe the stimulatory effect of sulfide on hydrogen evolution and to obtain evidence as to whether the nitrogenase or a hydrogenase-linked electron transport is influenced by sulfide.

Materials and Methods

Culture conditions

Nostoc muscorum (ATCC 7119) was grown in the medium of Allen and Arnon [10] with molecular nitrogen as the only nitrogen source. When cultured in 2 mM ammonia, K₂HPO₄ was increased to 8 mM. Culture conditions were described previously [5]. The cells were harvested in the logarithmic growth phase (usually after 3 days) and concentrated by sedimentation to a final chlorophyll content of 8 to 12 $\mu\text{g/ml}$ cell suspension which was equivalent to a packed cell volume (= pcv) of 6 to 10 $\mu\text{l/ml}$ cell suspension.

Assays

10 ml of the cell suspension concentrated as above was pipetted into 36-ml incubation vessels and sealed (Suba Seal, Freeman and Co., Barnsley, UK). To measure light-induced hydrogen evolution and acetylene reduction the vessels were flushed with pure argon; for acetylene reduction 10% C₂H₂ (v/v)

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was injected at start. For hydrogen-uptake determinations, the vessels were gassed with dinitrogen and 1% H_2 (v/v) was injected at start of the assay. The samples were then incubated under shaking in the light at 25 °C. Unless stated otherwise, light intensity was 7 W/m² (tungsten bulbs) to ensure linear rates. When indicated, sodium sulfide (from a 100 mM freshly prepared stock solution) and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) were added at the beginning of the test. A gas chromatograph from Hewlett-Packard (Mod. 5880A) with thermal-conductivity detector was used for hydrogen determination and a Varian aerograph (mod. 940) with flame-ionization detector for determination of ethylene.

Sulfide was measured by methylene-blue formed from 2-dimethyl-*p*-phenylenediamine, with ferric ions as catalyst, measured at 670 nm [11].

Fluorescence

Cell samples flushed with argon were incubated with and without 1 mM Na_2S in the light (7 W/m²). After different time intervals, samples were diluted with mineral medium to a final chlorophyll content of 2 µg/ml and placed into an open standard cuvette, which was incubated for 5 min in the dark prior to fluorescence measurements. Exciting light was provided by an 8 mW HeNe laser (632.8 nm; from Spectra Physics, Mt. View, CA, USA) with a light intensity of 15 W/m². Fluorescent light at the cuvette was measured from a 90° angle to the exciting light after having passed through a 680-nm cut-off filter (Schott, Mainz). The emitted light was amplified by a photomultiplier (Emi-Gencom, Plainview, NY, USA). The data were stored by a transient recorder (Datalab, Mitcham, Surrey, England, mod. DL 901) before plotting. Each measurement was done in triplicate with separate samples.

Results and Discussion

1. Sulfide-stimulation of hydrogen evolution

As shown in Fig. 1, incubation with sulfide affected both hydrogen metabolism and dinitrogen fixation, the latter measured as acetylene reduction. During the first 3 h, hydrogen evolution (part A) was not stimulated by the presence of 1 mM sulfide given at start. It increased after a 3-h lag phase and

reached an optimum rate of about 25 µmol H_2 /ml pcv × h as compared to a control rate of about 5 without sulfide. These data were calculated from the slopes of the curves between the 8th and the 12th h of incubation. The sulfide-stimulated rate was stable for 20 h. Acetylene reduction exhibited a similar inhibition at the beginning of incubation with 1 mM sulfide (Fig. 1B), but thereafter, the activity of the sample did not surpass that of the sulfide-free control.

In the presence of the uncoupler CCCP, neither substantial hydrogen evolution or acetylene reduction was seen (Fig. 1A, B). Furthermore, *Nostoc* grown in a medium containing 2 mM ammonia and not exhibiting heterocyst formation and dinitrogen fixation did not evolve hydrogen, whether or not sulfide was present (data not shown). These are compelling evidences that nitrogenase is involved in hydrogen evolution.

Two possible pathways of H_2 uptake in cyanobacteria have been reported [2, 12]. One is the oxyhydrogen reaction and the second hydrogen-supported nitrogenase activity. In our study, hydrogen uptake was measured under nitrogen atmosphere, because we found higher hydrogen-uptake activity than under argon. Since some oxygen generated by photosynthesis was present in the samples, we assume that both pathways of hydrogen uptake are operative under nitrogen atmosphere.

With sulfide present, hydrogen uptake was severely inhibited (Fig. 1A). This is seen by a hydrogen evolution (▲—▲) exhibited during the hydrogen-uptake assay within the first 8 h of sulfide incubation. Adding these evolution data and the (numerical) uptake figures of curve Δ—Δ, the gross hydrogen-evolving capacity of the cells is obtained, which has been drawn as curve ×—×. Most interestingly, this slope is very much identical to that of the sulfide-stimulated hydrogen production under argon (○—○). Since nitrogenase itself is not activated by sulfide (Fig. 1B), we take this as evidence that sulfide affects a hydrogenase-linked pathway which feeds hydrogen back to the nitrogenase or possibly also to oxygen. However, it must be emphasized that the hydrogenase reaction itself is not affected by sulfide. Partially purified hydrogenase from *Nostoc muscorum* was not influenced by 1 mM sulfide when hydrogen evolution was measured with dithionite-reduced methylviologen as electron donor (for preparation and assay see [13];

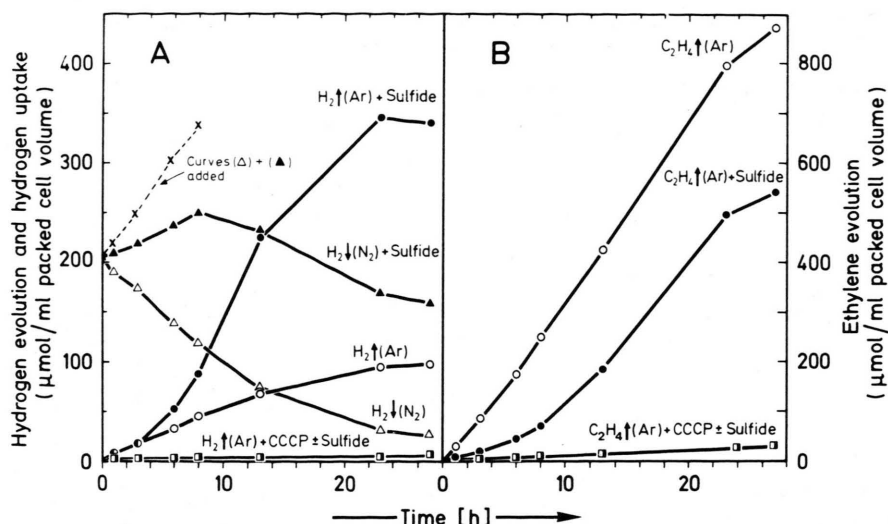


Fig. 1. Ethylene evolution, hydrogen evolution and uptake by *Nostoc muscorum* with or without 1 mM Na_2S present at start. (A) Hydrogen evolution under argon minus ($\circ-\circ$) and plus ($\bullet-\bullet$) sulfide; hydrogen uptake (and evolution) under nitrogen minus ($\Delta-\Delta$) and plus ($\blacktriangle-\blacktriangle$) sulfide; $\square-\square$, hydrogen and ethylene evolution with 5 μM carbonylcyanide-*m*-chlorophenylhydrazine (CCCP) present, plus or minus sulfide. Curve $\times-\times$ represents the addition of numerical values of curves $\Delta-\Delta$ and $\blacktriangle-\blacktriangle$ (representing gross hydrogen-evolving capacity of the cells). (B) Ethylene evolution minus ($\circ-\circ$) and plus ($\bullet-\bullet$) sulfide; $\square-\square$ ethylene evolution with 5 μM CCCP, plus or minus sulfide.

the rate was $12 \mu\text{mol H}_2/\text{mg protein} \times \text{h}$). The assumption that sulfide inhibits a hydrogenase-linked electron-transport chain is further corroborated by the data of Table I. Hydrogen evolution and uptake strongly depended on cultivation conditions (the exact parameters are just under investigation). Two *Nostoc* cultures were measured. Sample (A) exhib-

ited hydrogen uptake and sample (B) a small hydrogen evolution indicating little H_2 -uptake activity vs. (A) under conditions of the hydrogen-uptake assay of col. 2 (to measure the oxyhydrogen reaction, i.e. with an Ar-H_2 atmosphere and some oxygen present (generated by photosynthesis)). With both samples, addition of sulfide led to almost identical hydrogen-evolution data in cols. 1 and 2. This reflects a substantially higher degree of sulfide stimulation with sample (A) (factor 17) vs. sample (B) (factor 3).

The strong inhibition of hydrogen production as well as acetylene reduction during the first hours of sulfide incubation apparently is caused by the electron-donating system. Therefore, the influence of sulfide on photosynthetic electron transport was checked in more detail in sections 3 to 5.

2. Influence of light intensity

All reactions described were strictly light-dependent except for hydrogen uptake which – in part – is caused by dark respiration [14]. This is also true with sulfide present. The effects described above were observed with a light intensity of 7 W/m^2 which was generally applied to ensure linear rates for longer experimental times. Higher light intensi-

Table I. Hydrogen evolution, hydrogen uptake, and ethylene evolution by *Nostoc muscorum*. Rates in $\mu\text{mol}/\text{ml}$ packed cell volume $\times \text{h}$.

Species, addition	(1) Hydrogen evolution	(2) Hydrogen uptake	(3) Ethylene evolution
(A) <i>Nostoc muscorum</i>			
(-) sulfide	1.3	2.1	23.2
(+) sulfide	22.5	-22.6	17.3
(B) <i>Nostoc muscorum</i>			
(-) sulfide	7.2	-1.8	29.3
(+) sulfide	21.8	-19.8	20.3

Rates were measured after 6 h of incubation with and without 1 mM sulfide present at start. The assay of col. 1 had an argon atmosphere, that of col. 2 argon + 1% H_2 (v/v), col. 3 argon + 10% C_2H_2 (v/v). In all samples, some oxygen was present due to photosynthesis, allowing for H_2 uptake in the assay of column 2, first line. Under the conditions of this hydrogen-uptake assay, hydrogen evolution is denoted by (-).

Table II. Effect of sulfide on hydrogen and ethylene evolution at different light intensities by *Nostoc muscorum*.

Light intensity, additions	Hydrogen evolution	Ethylene evolution
3.5 W/m ² ; (+) sulfide	1.8	1.6
17.5 W/m ² ; (+) sulfide	10.1	9.6
35 W/m ² ; (+) sulfide	22.9	21.8
35 W/m ² ; (–) sulfide	2.3	37.2

Rates are given in $\mu\text{mol/ml pcv}\times\text{h}$ and were calculated from the data obtained between the 8th and 12th hour after start of incubation with 1 mM sulfide. Light intensities below 35 W/m² were adjusted with grey filters (Schott, Mainz).

ties increased sulfide-stimulated hydrogen evolution as well as acetylene reduction (Table II). As seen further, the (molar) ratio of hydrogen evolved to acetylene reduced was about 1 in the presence of sulfide. It was found to be about 0.1 without sulfide present (last line).

3. Dependence on sulfide concentration

This is shown in Fig. 2. 0.1 mM sulfide appears to be advantageous for hydrogen evolution during a 6-h incubation period (part A). Acetylene reduction decreased steadily when raising the sulfide content of the samples (part A). After 23 h, however, the effect on acetylene reduction was alleviated and stimulation of hydrogen evolution by sulfide was shifted to higher sulfide concentrations (part B).

These results are explained by the experiments of Table III. The sulfide-stimulated hydrogen produc-

Table III. The effect of sulfide on hydrogen and oxygen evolution by *Nostoc muscorum*.

Gases evolved; additions	Incubation time		
	1 h	6 h	14 h
	– $\mu\text{mol/ml}$ packed cell volume \times hour –		
1. Hydrogen evolution, control	6.3 (174.9)	5.2 (81.1)	4.9 (13)
2. Hydrogen evolution + sulfide	6.7	9.1	25.1
3. Oxygen evolution + sulfide	11.7	20.2	68.6
4. Sulfide concentration	0.05 mM	0.33 mM	0.16 mM
5. Sulfide concentration + 5 μM DCMU	0.75 mM	0.55 mM	0.35 mM

Cells were incubated under argon; sulfide concentration 1 mM at start of experiment. Data in parentheses (line 1) are oxygen evolution rates of the control, showing a decrease due to exhaustion of bicarbonate during the 6- and 14-h incubation time. Rates were determined over 1 h in col. 1 and between the 4th to 6th hour and 12th to 14th hour of incubation in cols. 2, 3, respectively.

tion is shown again in order to compare it with oxygen evolution. As seen in line (3), high oxygen-evolution rates showed up at the end of the incubation time indicating that the inhibition by sulfide was reversible. Photosynthetic oxygen evolution, though partially inhibited, caused a decrease of the sulfide concentration (line 4); consequently, presence of DCMU slowed down the decrease of sulfide (line 5). Therefore, the recovery of acetylene reduction and the shift of the optimum hydrogen evolution to higher sulfide concentrations with

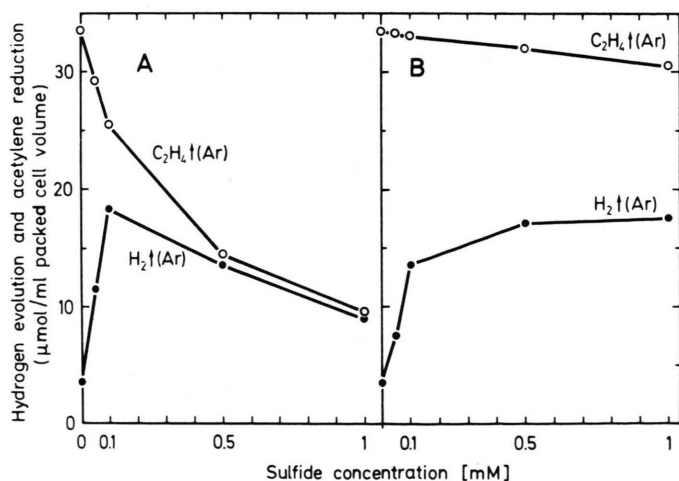


Fig. 2. *Nostoc muscorum*: dependence of hydrogen evolution and acetylene reduction on sulfide concentration at start of incubation. (A) After 6 h of incubation: hydrogen evolution (●-●), acetylene reduction (○-○). (B) After 23 h of incubation: hydrogen evolution (●-●) and acetylene reduction (○-○).

longer incubation times (Fig. 2B) are due to exhaustion of sulfide. Furthermore, the data of recovered oxygen evolution of line (2) indicate that oxygen does not inhibit the sulfide-stimulated hydrogen evolution.

4. Effects of sulfide on photosynthetic electron flow

Photosynthetic NADP reduction is inhibited by sulfide. Using *Nostoc* spheroplasts (see [15] for methods), the rate is lowered by 30% with 0.2 mM and 67% with 1 mM sulfide. Control rate was 60 μmol NADP reduced/mg Chl \times h. Fig. 3 demonstrates the possible site of inhibition. Part A exhibits the control fluorescence signal having a much

longer time course (0 to 4 s) with blue-greens than found with eukaryotes. The incubation time (2 to 13 h) of the control led to a strong decay of the P- and to some reduction of the I-(intermediate) level. Also the DCMU-induced fluorescence increase was lowered by longer incubation times. These effects, indicative of impaired electron transport, apparently are caused by the long illumination together with exhaustion of bicarbonate. The opposite was found with sulfide present. At start of incubation (2 h), a very low I-level and no P-maximum was seen, and no increase of fluorescence was possible with DCMU. Exhaustion of sulfide during incubation, however, led to a slow recovery of the I- and P-levels, the first regaining the height of the control, the latter remaining somewhat below. The time course of the fluorescence signal remained somewhat extended. This finding is interpreted as being due to an effect of sulfide at the oxidizing side of photosystem II, preventing electron donation to the reaction center, as was reported for *Aphanotece* and *Synechococcus*. No reversibility was shown [9]. In our experiments, the main inhibitory sulfide effect appears to be reversible, although an additional (irreversible) influence on the redox carriers between the photosystems cannot be excluded.

5. Incubation time; effect of DCMU

Hydrogen, oxygen, and ethylene formation were measured with either DCMU and/or sulfide present and with aliquots from which these additions had been removed by washing (Table IV). Apparently, removal of DCMU was incomplete, since photosynthesis could not be restored to the control level (line 2, col. B), as achieved with the sulfide-treated sample (line 3). Hydrogen evolution, however, was again drastically stimulated (col. A). This implies that the increased hydrogen evolution was not induced by *prolonged* sulfide incubation, but by the presence of a remaining sufficient sulfide level, low enough as not to prevent photosynthetic electron flow, but so high as to exert its effect on hydrogenase. Furthermore, as demonstrated by line (4), DCMU has little effect on the sulfide-induced stimulation, since samples containing inhibitor and sulfide exhibited increased hydrogen production after washing.

Summarizing then, two effects of sulfide showing strong interaction had to be elucidated. (1) Inhibi-

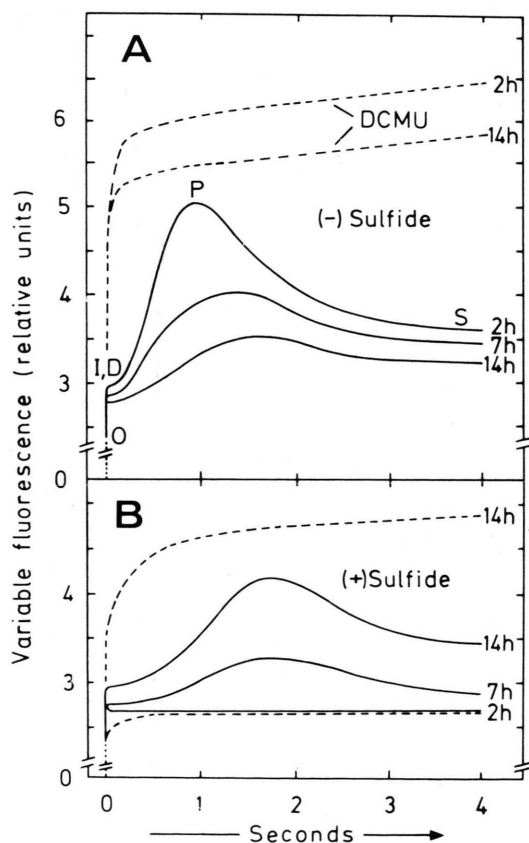


Fig. 3. Time course of chlorophyll-a fluorescence signals of *Nostoc muscorum* at different times (2, 7, 14 h) after starting incubation in the light without sulfide (A) or with 1 mM sulfide (B). DCMU concentration was 5 μM , the upper curve is the tracing of a 2-h incubation sample, the lower one was obtained after 14 h of incubation.

Table IV. Hydrogen, oxygen, and ethylene evolution by *Nostoc* before and after removal of DCMU and sulfide (in $\mu\text{mol}/\text{ml}$ packed cell volume \times h).

Additions	A Hydrogen washing ^a		B Oxygen washing ^a		C Ethylene washing ^a	
	without	with	without	with	without	with
1. Control	2.95	3.9 ^b	33.4	64.0 ^b	20.3	23.7
2. (+) 5 μM DCMU	5.7	0.9	0	26.4	12.2	11.8
3. (+) 0.5 mM sulfide	9.3	10.9	28.8	64.7	8.2	14.9
4. (+) 0.5 mM sulfide, 5 μM DCMU	3.1	7.2	0	20.2	2.7	7.3

All samples were kept under argon.

^a Samples with additions were incubated for 6 h in total and the rates determined between the 5th and 6th hour of incubation, either without or after washing. Washing was done twice with nutrient medium obtained from the supernatant of the control culture.

^b During the long incubation time, bicarbonate was exhausted leading to decreased photosynthesis (col. B). Therefore, oxygen- and even hydrogen-evolution rates (col. A) of the control were raised by adding fresh medium after washing, which contained bicarbonate.

tion of photosynthetic electron transport causing depletion of electrons needed for nitrogenase either to evolve hydrogen or reduce acetylene (see *e. g.* Table III). (2) Although less electrons were available with appropriate sulfide concentrations present, hydrogen evolution was speeded up, since the counteracting uptake-hydrogenase activity apparently was blocked by sulfide and hydrogen evolution on the whole yielded increased output even

under limited electron supply to nitrogenase (see Fig. 2A).

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