Maximizing Hydrogen Production by Cyanobacteria

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Z. Naturforsch. **63c**, 226–232 (2008); received October 31/December 5, 2007

When incubated anaerobically, in the light, in the presence of C_2H_2 and high concentrations of H_2, both Mo-grown *Anabaena variabilis* and either Mo- or V-grown *Anabaena azotica* produce large amounts of H_2 in addition to the H_2 initially added. In contrast, C_2H_2-reduction is diminished under these conditions. The additional H_2-production mainly originates from nitrogenase with the V-enzyme being more effective than the Mo-protein. This enhanced H_2-production in the presence of added H_2 and C_2H_2 should be of interest in approaches to commercially exploit solar energy conversion by cyanobacterial photosynthesis for the generation of molecular hydrogen as a clean energy source.

Key words: Hydrogenases, Alternative Nitrogenases, Photobiological Hydrogen Production in Cyanobacteria

Introduction

Many cyanobacteria have the simplest nutrient requirements among all organisms. They thrive photoautotrophically solely in inorganic media, and N_2-fixing species do not even require combined nitrogen (nitrate, ammonia or organic nitrogen) for growth. Another characteristic feature of cyanobacteria is the ability to produce or consume molecular hydrogen. They possess two different enzyme complexes systems capable of catalyzing H_2-production. The first system, the nitrogenases, evolves H_2 in parallel with NH_4^+-formation (Newton, 2007). Of the three known closely related types of nitrogenases (the Mo-, V- and Fe-only enzymes), both the Mo- and the V-nitrogenase have been reported for cyanobacteria (Kentemich *et al*., 1988; Thiel, 1993; Boison *et al*., 2006). The V-nitrogenase is less effective than the Mo-enzyme in catalyzing the reduction of both N_2 and C_2H_2 but consequently produces more H_2 (Pau, 1991). The second system comprises the hydrogenases that catalyze the heterolytic cleavage of H_2 into 2 H^+ and 2 e^-. Cyanobacteria express two different Ni-containing hydrogenases (Tamagnini *et al*., 2002; Schütz *et al*., 2004): The uptake hydrogenase encoded by *hupL* and *hupS* is believed to be (mainly or exclusively) confined to heterocysts where it recycles the electrons lost as H_2 during the N_2-fixation process. Because it feeds electrons into the respiratory complex II (Eisbrenner and Bothe, 1979), H_2-uptake by this enzyme is physiologically unidirectional on thermodynamic grounds. The other enzyme, the bidirectional (reversible) hydrogenase, is NAD(P)H-dependent and is encoded by the *hoxEFUYH* gene cluster. Although its location in the cells has not been finally resolved, it might also function in the uptake of H_2 under physiological conditions. This enzyme can, however, catalyze a transitory outburst of H_2-production when cells are exposed to an excess of reductant, *e. g.* under sudden high light intensities.

Over the years, many attempts have been made to couple photosynthetic electron transport with H_2-producing enzymes, either hydrogenase or nitrogenase. Solar energy conversion to H_2 by such photosynthetic organisms would mean the generation of "clean" combustible energy. Due to the demand for alternative energy sources, there is a renewed interest in such systems at present (Pinto *et al*., 2002; Tamagnini *et al*., 2002; Cournac *et al*., 2004; Levin *et al*., 2004; Schütz *et al*., 2004; Vignais and Colbeau, 2004; Shestakov and Mikheeva, 2006). Because H_2 allows one to store large amounts of energy within small volumes, H_2 is the energy carrier of choice for the future. H_2-production by cyanobacteria has, however, not reached a commercially acceptable level, because the efficiency in the conversion of incident light to H_2 is only 1–2% (Yoon *et al*., 2006) or even lower in many publications, although claims for a solar energy conversion factor of some 7% have been made in the past (Mitsui and Kumazawa, 1977).

Also problematic is the prerequisite for commercial H_2-exploitation that sustained H_2-production be achieved on a long-time scale. In addition, the clean up of the cyanobacterial culture after the termination of H_2-production might not be easily achieved. A significant enhancement in H_2-production by cyanobacteria must accrue if the cost-benefit ratio is to become promising.

An observation made by us during laboratory student course experiments may help to enhance the cyanobacterial H_2-production rates. C_2H_2-reduction by heterocystous cyanobacteria is more resistant to exposure to O_2 when the cells are incubated with H_2. H_2-consumption by hydrogenases in a Knallgas-type reaction apparently removes O_2 from the vicinity of nitrogenase thereby preventing its denaturation by O_2 (Bothe *et al.*, 1977). In experiments designed to demonstrate this effect, we were surprised to find that the control assays with no O_2 present in the vessels had significantly higher H_2-concentrations at the end of the experiments than was initially added. This anaerobic H_2-production in intact cells in the presence of both H_2 and C_2H_2 will be characterized here for the two heterocystous cyanobacteria *Anabaena variabilis* and *Anabaena azotica* where the latter of which was grown with either Mo or V in the medium.

Materials and Methods

Organisms and their growth

Anabaena variabilis ATTC 29413 was purchased. *Anabaena azotica* FACHB-118 from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China was kindly supplied by Professor Dai Heping, Wuhan, P. R. China. The cyanobacteria were grown in BG11 medium without combined nitrogen (Rippka *et al.*, 1979) at 25 °C under continuous gassing with a mixture of air/CO_2 and with continuous illumination (light intensity approximately at the surface of the vessels 70 μE m^{-2} s^{-1}) as described by Neuer and Bothe (1985). To induce the expression of V-nitrogenase, no Mo but 10 μM V_2O_5 was added to the medium. No attempt was made to remove Mo from the water or from any other chemical.

Mo-grown *A. variabilis* had a heterocyst frequency of (5.2 ± 0.7)% (n = 18, 100 cells counted for each). The number of heterocysts in the filaments was higher with *A. azotica*; it averaged (8.7 ± 1.0)% (n = 9) for Mo- and (11.5 ± 1.1)% (n = 12) for V-grown cultures. *A. azotica* produced significantly more extracellular polysaccharides and had a two- to threefold lower growth rate than *A. variabilis*. The growth rate of *A. azotica* was not impaired by substituting Mo by V in the medium.

Activity measurements by gas chromatography

Cyanobacterial filaments were concentrated in the culture tubes by their rapid self-sedimentation after terminating gassing. The experiments were performed in 7.0 ml Fernbach flasks covered with gas-tight suba seals into which C_2H_2 (1 ml) and H_2 (amounts given in the abscissa of the figures) were injected with a syringe. Prior to these injections of both H_2 and C_2H_2, the vessels were made anaerobic by gassing with argon. The dashed or dotted part of the columns in Fig. 1 indicates the amount of H_2 injected into the vessels immediately determined after the injection of the gases. Because H_2 had to be injected against the argon pressure in the vessels, H_2 did not proportionally increase with the milliliters of H_2 added by the syringe. The term "complete" in the figures means that argon was substituted by H_2 during the gassing procedure. The gas phase in the vessels then had a pressure of approximately 1 bar of H_2 that, however, did not prevent a further generation of H_2 by the cells against this pressure. The experiments were performed with 3 ml of cyanobacterial suspensions [total amount 0.035 mg chlorophyll, determined as described by Biggins (1967)] with the Fernbach flasks rotating on a horizontal shaker at 20 °C and a light intensity at the vessels of about 300 μE m^{-2} s^{-1}.

H_2 was quantified by gas chromatography using a Perkin Elmer 8500 gas chromatograph equipped with a thermal conductivity detector and a molecular sieve 5 Å column (45/60 mesh, 2 m × 1/8 inch). For C_2H_2-reduction, the C_2H_4 formed was also determined by gas chromatography using a flame ionization detector and a Carbosieve SII column (100/200 mesh, 10 feet × 1/8 inch). For further details see Kentemich *et al.* (1988).

Reproducibility of the experiments

All experiments presented in the figures and tables have been performed at least three times with the same outcome. However, the specific activities were variable from experiment to experiment, as known for physiological assays. The specific activi-

ties depend on the physiological state of the cells (batch cultures have been used; cells had to be concentrated prior to the start of the experiments which could hardly be standardized; the time needed for preparing the assay was variable). The content of cyanobacterial cells in the vessels varied by a factor of 1.5–2 from one experiment to the next. Therefore only representative data can be given here as in all other previous publications from this laboratory on this subject.

Results

When *A. variabilis* or *A. azotica*, either Mo- or V-grown, were incubated anaerobically in the light in the presence of high concentrations of both H_2 and C_2H_2 for 4 h, cells formed significantly more H_2 than was added at the start of the experiments. Rates of this additional H_2-formation were roughly ten times higher than the C_2H_2-reduction activities (Table I). The greater the amount of H_2 initially added to the assay vessels, the greater was the amount of additional H_2 produced. This was the case for both V- and Mo-grown *A. azotica* and also for Mo-grown *A. variabilis* at the higher H_2-concentrations (Fig. 1). This formation of additional H_2 proceeded linearly for at least 2 h (Fig. 2 for Mo- and V-grown *A. azotica*, same result for Mo-grown *A. variabilis*, not shown). The formation of additional H_2 was more obvious when the assays were performed at lower light intensities and with smaller amounts of the cells in the reaction vessels, as in the current experiments (comparative data not shown). Without adding H_2 to the vessels, net H_2-production was less than

Assay condition	C_2H_2-reduction [μmol C_2H_4 formed/ h · mg chlorophyll]	H_2-formation [μmol H_2 formed/ h · mg chlorophyll]
Anabaena azotica		
V-culture, without H_2	3.4	0.6
V-culture, plus H_2	3.3	40
Mo-culture, without H_2	4.0	0.1
Mo-culture, plus H_2	1.8	9
Anabaena variabilis		
Mo-culture, without H_2	7.8	0.5
Mo-culture, plus H_2	3.3	24

Table I. C_2H_2-reduction and H_2-formation activities by *Anabaena azotica* and *A. variabilis*.

The experiments were performed under argon in 7.0 ml Fernbach flasks into which C_2H_2 and H_2 (each 1 ml) were injected. C_2H_4-formation was determined after 1 h and H_2-production after 4 h. For other details see Materials and Methods.

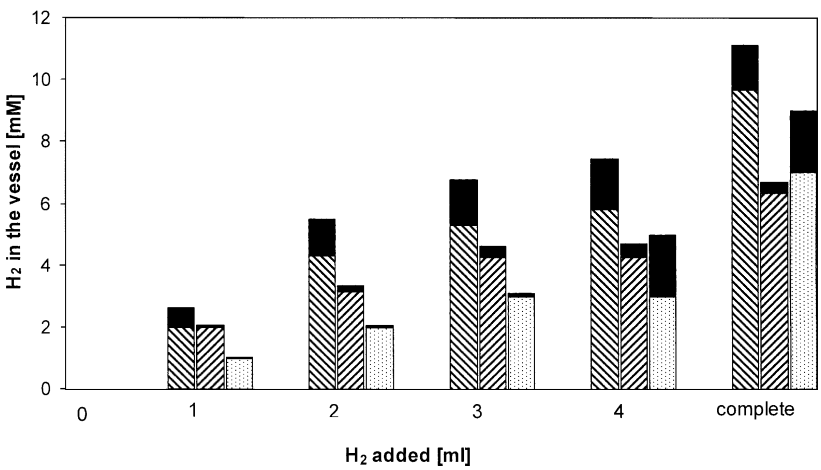

Fig. 1. H_2-production by *Anabaena azotica* (V- or Mo-grown) and *A. variabilis*. The black part of the columns indicates the concentration of H_2 formed per 4 h and mg chlorophyll in the vessels, the dashed or dotted part of the columns the amount of H_2 added to the vessels by syringes and determined by gas chromatography at the start of the experiments. Columns with dashes to the left, *A. azotica* V-grown; columns with dashes to the right, *A. azotica* Mo-grown; columns with dots, *A. variabilis* Mo-grown. Complete means gas phase H_2 (about 1 bar).

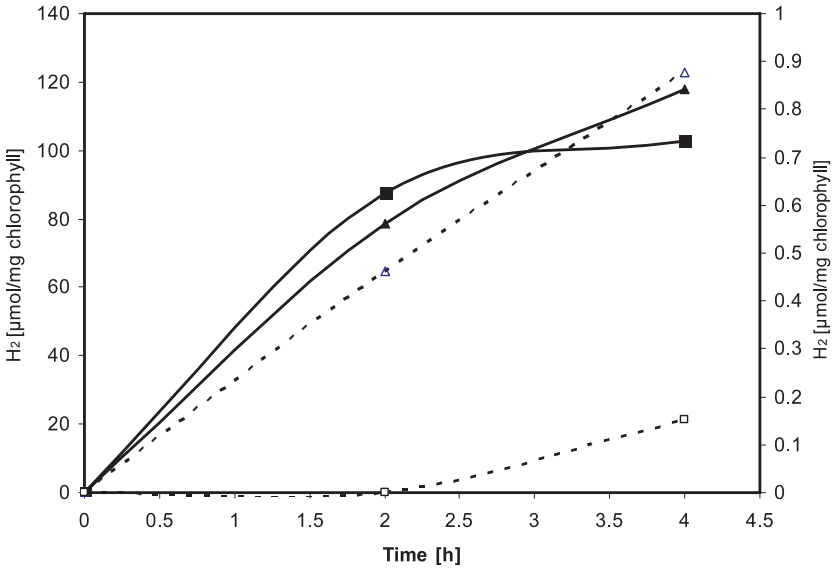

Fig. 2. Kinetics of H_2-formation by Mo- and V-grown *Anabaena azotica*. Solid symbols and lines indicate assays in Fernbach flasks supplemented with 1 ml H_2; the appropriate rate scale is shown on the left ordinate. Dashed lines and open symbols indicate assays without H_2 supplementation; the appropriate rate scale is shown on the right ordinate. In this experiment, H_2-production was atypically as high in Mo- as in V-grown cultures which happened with approximately one out of ten cultures. Triangles, Mo-grown culture; squares, V-grown culture.

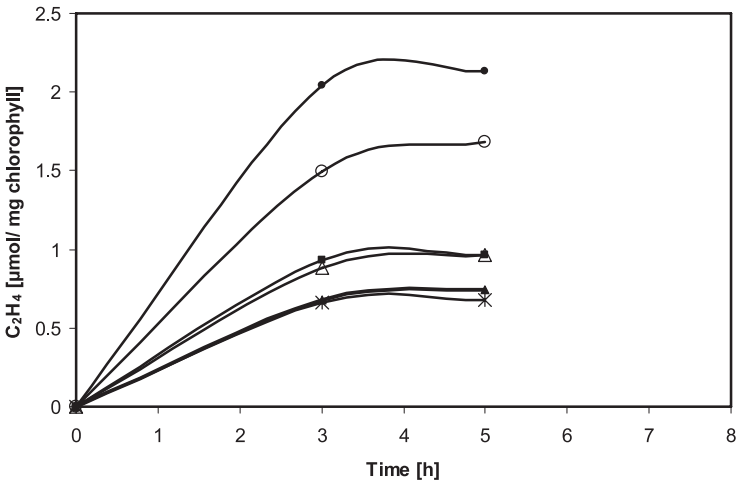

Fig. 3. Inhibition of C_2H_2-reduction by increasing concentrations of H_2 added to the assays, using Mo-grown *A. azotica*. Top line, no H_2 injected into the vessels; the following lines, 1, 2, 3, and 4 ml H_2 injected; the bottom line, 100% H_2 gas phase. The inhibition pattern was the same for V-grown *A. azotica* and for Mo-grown *A. variabilis* (not documented).

1.0 μmol/h · mg chlorophyll (Table I and right ordinate of Fig. 2), in accordance with earlier determinations (Bothe *et al.*, 1978).

In the same experiments, C_2H_4-formation showed a steady decline as increasing amounts of H_2 were added (Fig. 3) and stabilized at about 30% of the original H_2-free activity under 100% H_2. A concentration of 6 mM H_2 in the assays decreased C_2H_4-formation by about 50%, irrespective of whether the cells were Mo- or V-grown.

Both H_2-formation (in addition to the H_2 added) and C_2H_2-reduction were greatly stimu-

Table II. C_2H_4- and H_2-formations by vanadium-grown *Anabaena azotica*: Effects of inhibitors.

Assay condition	C_2H_4-formation [μmol/h · mg chlorophyll]	H_2-formation (μmol/h · mg chlorophyll)
Control without H_2	2.4	0.1
H_2 alone	1.2	34.5
H_2, 40 μM DCMU	0.5	13.2
H_2, 80 μM DCMU	0.4	11.3
H_2, 6 μM CCP	1.0	17.9
H_2, 12 μM CCP	0.8	15.8
H_2, 6 μM FCCP	0.3	12.4
H_2, 12 μM FCCP	0.2	9.0
H_2, vessels kept in darkness	0.0	6.0
H_2, N_2 instead of C_2H_2	–	0.0

The experimental conditions were as for Table I but 3 ml H_2 were injected to the vessels this case.
DCMU, dichlorophenyl dimethyl urea, first dissolved in 25% methanol, then diluted tenfold with water to give the final concentration in the vessel; CCP, carbonyl-cyanide-phenylhydrazone; FCCP, carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone (*p*-CF_3-CCP).

lated by light and were sensitive to inhibitors of photosynthetic electron transport (Table II). The herbicide DCMU (dichlorophenyl dimethyl urea), which blocks at the acceptor side of photosystem II, affected both additional H_2-formation and C_2H_2-reduction to approx. the same extent. Uncouplers also severely affected both activities with FCCP (carbonyl-cyanide-phenylhydrazone) being more effective than CCP (carbonyl-cyanide-*p*-trifluoromethoxyphenyl-hydrazone). Because the activities of nitrogenases, but not of hydrogenases, are energy (ATP)-dependent, the additional H_2-production must have been produced mainly by nitrogenase. No additional H_2-formation was observed when N_2 replaced C_2H_2 as the nitrogenase substrate (Table II). H_2-production was also not seen in nitrate-grown *A. variabilis* when nitrogenase was not expressed (not documented).

Discussion

In the presence of N_2, cells might be in balance with respect to their C/N ratio, and this balance is not affected by the addition of H_2. The H_2-gas formed in parallel with ammonia production in nitrogenase catalysis is immediately recycled by hydrogenases. This is manifested by rather little net production in intact cyanobacterial cells. In the presence of C_2H_2, however, high concentrations of H_2 cause a disturbance of this C/N ratio, possibly due to the fact that reductants generated photosynthetically cannot be properly utilized anymore to meet the N-demand of the cells. C_2H_2-reduction is diminished, and the excess of reductants is disposed of to reduce protons. This inhibition of C_2H_2-reduction by H_2 and the parallel production of additional H_2 in the presence of high concentration of H_2 in the assays are apparently hitherto unreported observations. It has, however, been reported for bacteroids that high concentrations of H_2 inhibit N_2-fixation, and that H_2-recycling by hydrogenase prevents the build-up of high inhibitory concentrations of H_2 formed during N_2-reduction in nodules (Dixon, 1972).

The high concentrations of H_2 and C_2H_2 unlikely affect nitrogenase itself. Hydrogen is a competitive inhibitor of N_2-reduction but not in the reduction of all other nitrogenase substrates including C_2H_2 (Burns and Hardy, 1975). In cyanobacteria, C_2H_2 (and CO) was described to inhibit the photosystem I or respiration-dependent uptake of H_2 (Bothe *et al.*, 1977) which could partly explain the enhanced H_2-production. However, this does not explain the effect of the high concentrations of H_2. The primary target of these high concentrations of H_2 remains obscure and speculative. In line with the present findings, a two- to fourfold increase of light-induced H_2-evolution was described for the cyanobacterium *Nostoc muscorum* preincubated under hydrogen and argon years ago. The parallel inhibition was, however, not detected (Scherer *et al.*, 1980).

The light stimulation and the results with the inhibitors indicate that reducing equivalents for H_2-formation are produced mainly photosynthetically. The reductants generated in the light might be disposed by H_2-production via ferredoxin and nitrogenase to avoid an overreduction in the cells and an imbalance in the C/N ratio. A direct coupling of H_2-production with reduced ferredoxin and hydrogenase(s) can be ruled out, since both cyanobacterial hydrogenases (uptake and bidirectional enzyme) do not utilize this electron carrier (Tamagnini *et al.*, 2002; Schütz *et al.*, 2004). It could be assumed that H_2-production comes from ferre-

doxin, FNR [NAD(P)H:ferredoxin oxidoreductase], $NAD(P)^+$ and bidirectional hydrogenase. Such an activity should, however, be insensitive to uncouplers. Such an interpretation can also not explain that H_2-formation is higher in V- than in Mo-grown cells. To a minor extent, reducing equivalents for H_2-production may also come from carbohydrate metabolism, since the inhibitors of photosynthesis do not completely block the H_2-production. Reductants generated during fermentative degradations of reserve material may be disposed of via pyruvate:ferredoxin oxidoreductase, FNR and bidirectional hydrogenase. Cyanobacteria are known to rapidly switch over to fermentation when exposed to the dark (Stal and Mozelaar, 1997). This smaller part of the H_2-formation would then be produced by hydrogenase(s) and not by nitrogenase.

As first demonstrated for *Azotobacter vinelandii*, alternative nitrogenases produce more H_2 than the Mo-enzyme (Bishop and Joerger, 1990). This is also seen in the cyanobacteria *A. variabilis* (Kentemich *et al*., 1988) and *A. azotica* (present study) having expressed the V-nitrogenase. Compared with the V-enzyme, an even higher H_2-formation and a lower C_2H_2-reduction rate is seen with the Fe-only nitrogenase (Bishop and Joerger, 1990). Up to this day, an Fe-only nitrogenase has not been described for cyanobacteria, and the V-nitrogenase is known only for few isolates. An Fe-only nitrogenase is known for the photosynthetic bacterium *Rhodobacter capsulatus* (Newton, 2007) where it could drive photosynthetic H_2-production, however not at the expense of H_2O as the electron donor. The occurrence of an Fe-only nitrogenase in cyanobacteria cannot be ruled out, although it is not found in the genome of *A. variabilis* and *Anabaena* 7120. Negative results of growth experiments performed in the absence of both Mo and V in the medium are not convincing, since it is not easy to obtain both Mo- and V-deficiency in cultures due to the low demands of cells for these elements. Giving an example outside of cyanobacteria, the Fe-only nitrogenase has now, unexpectedly, been detected in the plant growth promoting bacterium *Azospirillum brasilense* Sp245 from the analysis of its completely sequenced genome (Zhulin, 2007).

For maximizing H_2-production by cyanobacteria for potential applications, it is suggested to take *Anabaena variabilis* with an expressed V-nitrogenase. In contrast, *A. azotica* has higher amounts of heterocysts as the site of nitrogenase under aerobic growth conditions, but forms fairly large amounts of exopolysaccharides (slime) and grows slower than *A. variabilis*. Incubation of the cultures with high concentrations of H_2 and of C_2H_2 enhances H_2-formation, and this present observation can possibly be exploited for applications. For doing so, conditions still have to be optimized. Mutants defective in hydrogenases to prevent recycling of H_2 might also augment the photosynthetically driven H_2-production by cyanobacteria. Indeed, some years ago, we observed higher H_2-production rates in an *A. variabilis* mutant obtained by classical NTG mutagenesis which was affected in the recycling of H_2 produced by nitrogenase (Mikheeva *et al*., 1995). Mutants defective in either uptake, bidirectional or both uptake and bidirectional hydrogenase have been constructed for *Anabaena* 7120, and activity measurements indicated that only the mutant defective in the uptake hydrogenase produced more H_2 (Masukawa *et al*., 2002). Work with hydrogenase mutants from other cyanobacteria also showed increased H_2-evolution rates than the wild-types (Happe *et al*., 2001; Lindblad *et al*., 2002; Cournac *et al*., 2004; Yoshino *et al*., 2007). A mutant in *A. variabilis* having expressed the V-nitrogenase would be better suited to optimize H_2-production by working out the best culture conditions such as stability of the cultures for sustained gas production, best cell density or light intensity. The alternative approach to utilize a cyanobacterial hydrogenase will likely be less successful, since both enzymes mainly function in H_2-utilization. Other attempts consist in engineering a foreign hydrogenase and in coupling it with photoystem I for H_2-production. In such a recent approach, the membrane-bound enzyme from *Ralstonia eutropha* was coupled directly to a modified component (PsaE) of the acceptor side of the cyanobacterial photosystem I (Ihara *et al.*, 2006). The small H_2-formation rates obtained with this artificial system may reflect that all such approaches are still far away from potential applications.

Acknowledgement

The authors are indebted to Professor William E. Newton, Blacksburg, VA, USA for helpful comments and for amending the English of the manuscript.

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