Mass Spectrometric Analysis of N_2-Formation Induced by the Oxidation of Hydrazine and Hydroxylamine in Flash Illuminated Thylakoid Preparations of the Filamentous Cyanobacterium *Oscillatoria chalybea*

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In tobacco chloroplasts hydrazine-dependent dinitrogen formation measured by mass spectrometry as the consequence of short saturating light flashes is always linked to a substantial oxygen uptake (G. Renger, K. P. Bader, and G. H. Schmid, Biochim. Biophys. Acta **1015,** 288, 1990). However, in thylakoids of the filamentous cyanobacterium *Oscillatoria chalybea* this dinitrogen formation is not linked to an apparent O_2-uptake, even at the high concentration of 1 mM hydrazine. Whereas in tobacco chloroplasts Tris-treatment does not affect hydrazine dependent dinitrogen formation up to a concentration of 3 mM hydrazine, Tris-treatment of thylakoids of *O. chalybea* affects strongly both oxygen evolution and dinitrogen evolution under a single turnover flash as well as under ten flashes. In contrast to tobacco chloroplasts, the presence of hydrazine up to concentrations of 3 mM does not substantially affect photosynthetic O_2-evolution. The observed dinitrogen evolution is affected by DCMU regardless whether induced by a single turnover flash or by ten flashes, whereas in tobacco dinitrogen evolution and the O_2-uptake linked to it (which is not observed in the cyanobacterium) were clearly not affected by DCMU in the single turnover flash. In *Oscillatoria* the earlier described Photosystem II-mediated H_2O_2 formation and decomposition is influenced by hydrazine. In the presence of 300 μM hydrazine the usually present O_2-uptake leading to H_2O_2 formation appears diminished.

Introduction

In the filamentous cyanobacterium *Oscillatoria chalybea* photosystem II exhibits in comparison to that in higher plants a number of essential differences [1–4]. Thus, if photosynthetic oxygen evolution is analyzed as the consequence of short saturating light flashes, the observed oxygen evolution pattern differs from that described manyfold in the literature for *Chlorella* or higher plant chloroplasts [5]. In an *Oscillatoria* sequence the first flash and the second flash yield oxygen even after an extensive dark adaptation, an observation which was correlated to metastable (long-living) S_3 and also long living S_2 [1, 2]. In general the life time of the S-states seems to be increased in *Oscillatoria* [6] a property which is also observed in higher plants when the photosynthetic apparatus develops its structure and function during greening [7]. Thus, Franck and Schmid have shown that in the early stage of greening oat etioplasts exhibit photosystem II properties comparable to those of the phylogenetically old filamentous cyanobacterium *Oscillatoria chalybea*. Due to these properties it was possible to characterize the effect of reducing agents, such as hydroxylamine, on S_1, S_2 and S_3 by adding the inhibitor shortly after two flashes and by recording the oxygen flash sequence thereafter [8]. In *Oscillatoria* photosynthetic oxygen evolution under normal oxygen partial pressure of air always consists of two portions, one coming from water-splitting and the other from H_2O_2-decomposition [3]. The electrons for the H_2O_2-formation seem to originate from water, with the formation of H_2O_2 being due to the interaction of oxygen with one of the S-states [9]. In the present paper we investigate the effect of hydrazine and to a minor extent that of hydroxylamine, which according to the literature interact with the S-state system [10, 11] on the S-state system of *Oscillatoria chalybea*.

Abbreviations: Tris, Tris(hydroxymethyl-)aminoethane.

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Materials and Methods

The filamentous cyanobacterium Oscillatoria chalybea was obtained from the algal collection in Göttingen (F.R.G.) and cultured for the assays of the present paper on nitrate as the sole nitrogen source in the medium. Growth conditions and medium compositions have been described earlier [1].

Thylakoid preparations of Oscillatoria chalybea were prepared according to Bader *et al.* [1]. The mucoid layer on the cells was digested with glucuronidase (Boehringer, Mannheim) and the cell walls with lysozyme (Sigma) and cellulase (Kinki Yakult, Japan).

Tobacco chloroplasts were prepared from *Nicotiana tabacum* var. John William's Broadleaf (JWB) according to Homann and Schmid [12]. The freshly prepared chloroplasts were stored on ice before the measurements in the mass spectrometer.

The reaction mixture contained in a total of 2 ml tobacco chloroplasts corresponding to 70 µg chlorophyll and thylakoids of *Oscillatoria* corresponding to 40 µg chlorophyll, 30 mM KCl and 60 mM Tricine-NaOH (pH 7) and the additions of NH_2NH_2 or NH_2OH indicated in the figures.

Mass spectrometry was carried out with a modified magnetic sector field mass spectrometer type "Delta" from Finnigan MAT (Bremen, F.R.G.), which is an isotope ratio mass spectrometer equipped with a two-directional focussing device "Nier type I" [2]. The experimental set-up with the valve system is described in detail earlier [2].

Saturating light flashes of 8 µs duration were obtained from a xenon flash lamp (Stroboscope 1539 A of General Radio). The time between flashes was 300 ms.

^{15}N-Labeled hydrazine and hydroxylamine were purchased from IC-Chemicalien (München, F.R.G.).

^{18}O-Labeled water was purchased from Ventron (Karlsruhe, F.R.G.).

Results

The effect of increasing hydrazine concentrations on oxygen evolution and dinitrogen evolution in normal and Tris-washed thylakoid preparations of *Oscillatoria chalybea* is shown in Fig. 1. Several points are different when compared to tobacco chloroplasts [13]. First the apparent oxygen evolution in *Oscillatoria* thylakoids is not much affected by hydrazine concentrations up to 1000 µM, whereas in tobacco chloroplasts O_2-evolution is zero around 300 µM. Also in contrast to tobacco chloroplasts dinitrogen evolution under both the single turn-over flash or by a train of saturating flashes is sensitive to Tris-treatment (Fig. 1), whereas in tobacco chloroplasts it is not [13]. On the other hand, O_2-evolution in *Oscillatoria* thylakoids is sensitive to Tris-treatment (Fig. 1) as it

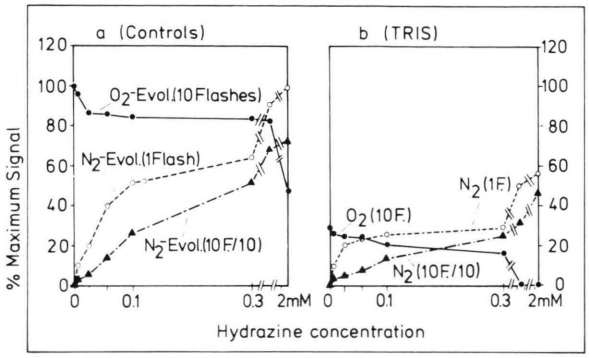

Fig. 1. Dependence of flash-induced $^{15}N_2$-formation on the $^{15}NH_2-^{15}NH_2$ concentration in normal and Tris-treated thylakoids of *Oscillatoria chalybea*. Dark-adapted samples were illuminated with either 1 flash or ten flashes. ▲—▲, $^{15}N_2$-evolution per flash in a train of ten flashes; ○—○, $^{15}N_2$-evolution after illumination with one flash; ●—●, photosynthetic $^{16}O_2$-evolution in a train of ten flashes.

should be and as is the case with tobacco chloroplasts. Dinitrogen evolution from hydrazine oxidation is linked in tobacco chloroplasts to a considerable O_2-uptake [13] whereas in *Oscillatoria* thylakoids no such uptake is observed (Fig. 2). The assay contained 25% $H_2{}^{18}O$ in its buffer system and was carried out in the presence of 100 µM hydrazine. The assay was in equilibrium with normal air. Under the assumption that the observed O_2-evolution comes exclusively from water-splitting the isotope ratios between masses 32, 34 and 36 should according to equation $32:34:36 = (1-\alpha)^2:2\alpha\,(1-\alpha):\alpha^2$ be for 32:34:36, with α being the atom fraction which is ^{18}O, 56.25:37.5:6.25. Fig. 2 clearly shows that the measured mass 32 peak exceeds manyfold the necessary value. Due to the fact that the observed ratio between masses 34 and 36 is 5, 9/1, which is close to the theoretical ratio, the observed $^{16}O_2$-evolution does not come

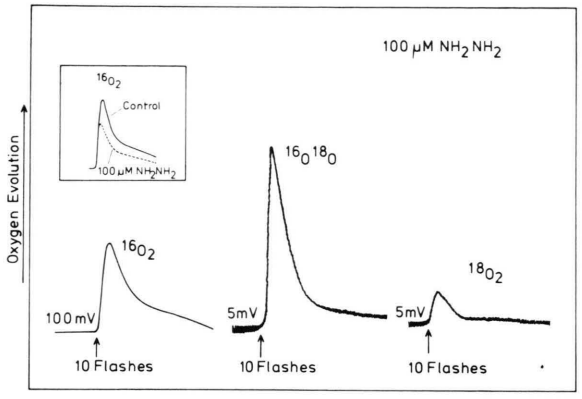

Fig. 2. Dioxygen evolution measured at mass 32 ($^{16}O_2$), 34 ($^{16}O^{18}O$) and 36 ($^{18}O_2$), respectively, as a consequence of illumination with ten flashes in a thylakoid preparation of *Oscillatoria chalybea* incubated with 100 μM NH_2NH_2 in an aqueous suspension containing 25% $H_2{}^{18}O$. In a control experiment with the same preparation in the absence of hydrazine and ^{18}O-labelled water the di-oxygen evolution at mass 32 ($^{16}O_2$) was measured (dashed line) to show the effect of hydrazine on O_2-evolution. The experiments shows that no apparent O_2-uptake is linked with hydrazine oxidation.

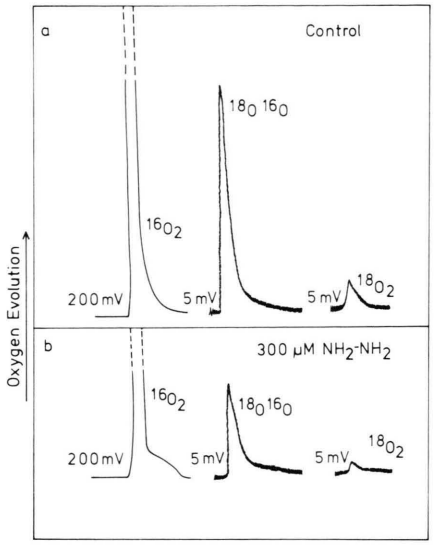

Fig. 3. Dioxygen evolution measured at mass 32 ($^{16}O_2$), at mass 34 ($^{16}O^{18}O$) and at mass 36 ($^{18}O_2$) as a consequence of illumination with ten flashes in a thylakoid preparation of *Oscillatoria chalybea*. The aqueous suspension contained 25% $H_2{}^{18}O$. Control experiment (a) and (b) assay incubated with 300 μM NH_2NH_2.

from $H_2{}^{18}O/H_2{}^{16}O$ splitting. In fact, the observed $^{16}O_2$-evolution is due to the photosystem II mediated H_2O_2-formation and decomposition described in detail earlier [3, 9]. Indeed, it was shown in this cyanobacterium that photosynthetic oxygen evolution measured as the consequence of a train of short saturating light flashes contains two portions, one coming from water-splitting proper and the other by interference of photosystem II with O_2 (*e.g.* of the gas phase), leading to H_2O_2 formation which is decomposed by the S-state system to give O_2, protons and electrons [3, 9]. Mass spectrometry shows that qualitatively O_2-evolution coming from water-splitting proper is in *Oscillatoria chalybea* inhibited by increasing concentrations of hydrazine (Fig. 3) just as in tobacco [13]. At a concentration of 300 μM hydrazine in *Oscillatoria* approx. 50% of the water-splitting activity has disappeared (Fig. 3) whereas at the same concentration oxygen evolution in tobacco is totally absent. Despite the first impression given by Fig. 2 dinitrogen evolution in *Oscillatoria* clearly depends on the oxygen partial pressure in the assay. Decreasing oxygen background in the assay decreases the observed dinitrogen signal (Fig. 4), hence the absence of an apparent oxygen uptake linked to dinitrogen evolution does not exclude the reaction of the $HN^{\circ}-NH_2$-radical whith oxygen [13]. It rather looks as if hydrogen peroxide decomposition was inhibited or its production lesser in the presence of hydrazine (Fig. 2). A close-up scrutiny of the oxygen signal at mass 32 in the presence and absence of 300 μM hydrazine reveals some marked shape

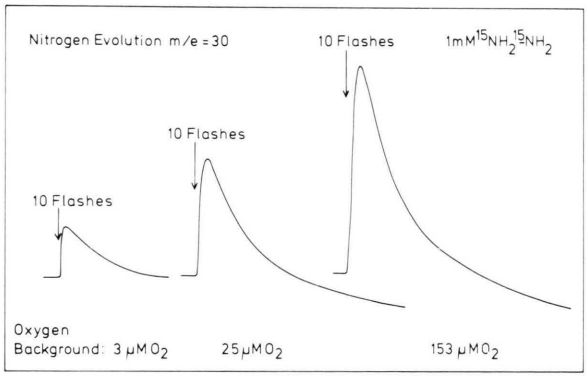

Fig. 4. $^{15}O_2$-Evolution in a thylakoid preparation of *Oscillatoria chalybea*, incubated with 100 μM NH_2NH_2 and illuminated with a train of ten flashes, in dependence on the O_2-content of the assay.

differences (Fig. 5). Oxygen evolution measured by mass spectrometry as the consequence of a train of 10 flashes (300 ms between flashes) in a single (cumulated) signal shows a respectable portion of delayed oxygen evolution seen as a signal shoulder in Fig. 5, which should correspond to the two digit shift of flash patterns in the presence of hydrazine or hydroxylamine [11, 14]. This delayed oxygen evolution is DCMU sensitive and it looks as if this portion of O_2 evolution was more sensitive than the not delayed one (Fig. 6). This means that the interaction of hydrazine with the donor side of photosystem II in *Oscillatoria* might be two-fold: part of the compound reacts with the S-state which decomposes in *Oscillatoria* H_2O_2 (probably with S_2, less probably S_3) or it reacts with H_2O_2 or the oxygen resulting from its decomposition in the way discussed earlier [9]. Formally hydrazine just takes the place of H_2O_2 (Fig. 2). The major effect of the compound consists in an interaction with the S-state system namely with that particular state which is the most reactive one to hydrazine, leading to a reduction of the S-state system and to the otherwise observed two digit shift of the O_2 evolution pattern in a train of saturating light flashes. As the peculiarity of the dark adapted S-state system in *Oscillatoria* is, in comparison to higher plants, the longer half-life time of all S-states, in particular that of the S_2 and S_3 states, the effect and the difference to tobacco chloroplasts [13] lies probably in this region.

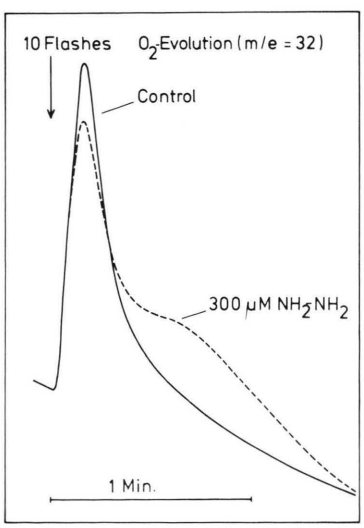

Fig. 5. Shape analysis of the O_2-evolution signal at mass 32 ($^{16}O_2$), obtained from an assay with thylakoids of *Oscillatoria chalybea*, incubated with 300 μM NH_2NH_2 and illuminated with a train of ten flashes. The assay contained 25% $H_2{}^{18}O$.

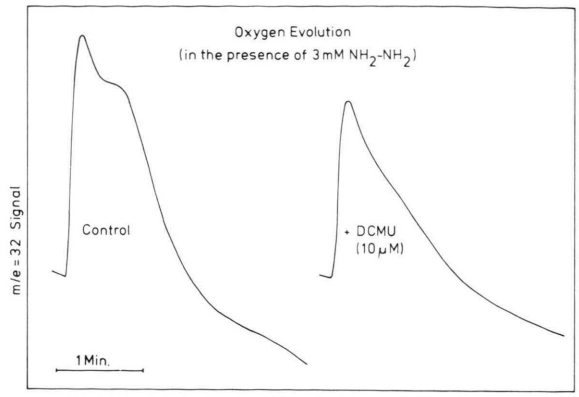

Fig. 6. O_2-Evolution signal at mass 32 ($^{16}O_2$) obtained from *Oscillatoria chalybea* thylakoids incubated with 3000 μM NH_2NH_2 and illuminated with a train of ten flashes. The assay contained no ^{18}O-labeled water. Same assay in the presence of 10^{-5} M DCMU.

The mechanism of dinitrogen formation from hydrazine and hydroxylamine oxidation of photosystem II of *Oscillatoria* clearly is the same as in tobacco. If equimolar amounts of ^{14}N-labelled and ^{15}N-labelled hydrazine are presented to the system, only masses 28 and 30 are observed (Fig. 7), the

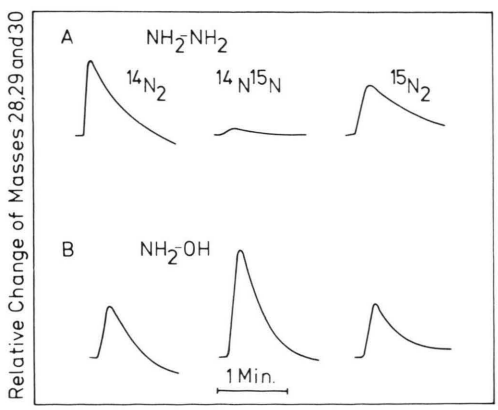

Fig. 7. Dinitrogen formation measured at mass 28 ($^{14}N_2$), 29 ($^{14}N^{15}N$) and 30 ($^{15}N^{15}N$) as the consequence of illumination with ten flashes in thylakoids of *Oscillatoria chalybea* incubated with an equimolar mixture of $^{14}NH_2$–$^{14}NH_2$ and $^{15}NH_2$–$^{15}NH_2$ (A – top) and of $^{14}NH_2OH$ and $^{15}NH_2O$ (B – bottom) at a concentration of 1000 μM hydrazine or hydroxylamine.

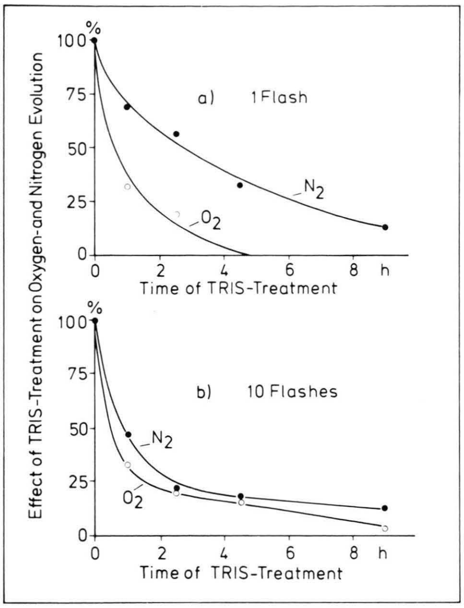

Fig. 8. Effect of the duration of treatment with 0.8 M Tris at pH 8 on the dinitrogen and photosynthetic dioxygen evolution in thylakoids of *Oscillatoria chalybea* incubated with 1000 μM NH_2NH_2 and illuminated a) with one single turnover flash; b) with a train of ten flashes.

mixed label is absent. If the same experiment is made with an equimolar mixture of $^{15}NH_2OH$ and $^{14}NH_2OH$, the mixed labeled ($^{15}N:::^{14}N$) nitrogen is the predominant species (Fig. 7). The mechanism therefore should be that of an univalent oxidation of NH_2OH and NH_2NH_2 as described for tobacco chloroplasts [13]. However, in *Oscillatoria* the major difference within this mechanism type is the fact that here the water oxidase seems to be involved or necessary for this type of oxidation, since dinitrogen evolution under the single turnover flash as well as under a train of 10 saturating light flashes is sensitive to Tris-treatment (Fig. 8).

Discussion

According to Joliot [15] and Kok [16] photosynthetic water splitting takes place *via* a sequence of 4 oxidation steps in which a manganese containing enzyme complex successively goes through 4 redox states, eventually leading to the splitting of water and the evolution of oxygen. This system of redox states has been termed S-state system [19]. In the transition from

$$S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4 \rightarrow O_2$$

(with a return arrow from S_4 to S_0)

no intermediate (such as an "oxygen precursor" or partially oxidized water) has been detected so far [2, 17] although some experimental evidence hints at a peroxidic state [18]. The observation that no reaction intermediate or even binding of bulk water to one of the S-states up to the S_3-condition should occur is disturbing and not easily acceptable from the thermodynamic point of view. As pointed out in earlier publications the water-splitting complex of the filamentous cyanobacterium *Oscillatoria chalybea* exhibits a number of peculiarities in comparison to that of higher plant chloroplasts [1–3]. The observed properties of the S-state system in *Oscillatoria* compare only in a defined state of chloroplast development, namely in an early greening stage to that of higher plants. In both conditions the S-state system is characterized by a longer life-time of the S-states measurable as a much slower S-state deactivation than usual. This is particularly striking with respect to the S_2- and S_3-states which are states that in the normal higher plant situation decay within a few seconds whereas corresponding states in *Oscillatoria* or greening oat chloroplasts are much longer living. Thus, it is observed that the S_2-state has a half-life time of 1 min and a portion of the S_3-state is stable for many minutes (*i.e.* metastable). There is no doubt that the observed O_2-evolution pattern in *Oscillatoria* as the measured consequence of short saturating light flashes fits all requirements of the Kok model, with the peculiarity that an *Oscillatoria* pattern gives much better fits in the four state Kok model than a *Chlorella* pattern does [1]. This is to say that the measured O_2-signals in *Oscillatoria* surely measure the S_2- and S_3-state and nothing else. Already in this situation it is striking that S_3 might be a condition which is very little reactive and does certainly not react with oxygen, otherwise it would not be metastable. As the search for intermediates has not been successful so far [2, 17] a comparison of the endogenous reactivity of the S-states in *Oscillatoria* and higher plants, together with the reactivities of the S_1-state towards exogenous reagents like hydrazine or hydroxylamine promisses some interesting insights. If one starts out from the point of view that the *Oscillatoria* properties are also seen in higher plant chloro-

plasts under special conditions such as greening [7] or at low temperatures [19], the comparison of the endogenous S-state properties within *Oscillatoria* to a certain extent might reflect the regular situation amongst the S-states in the much faster higher plant system: In all systems S_1 is the least reactive state as it is dark stable; S_0 in the dark slowly oxidizes to S_1 and the major part of S_2 decays directly to S_1 [20]. The S_2 decay kinetics in relation to the other S-states are alike in higher plant chloroplasts and in *Oscillatoria,* with the difference, as said above, that S_2 is much longer lived in *Oscillatoria.* However, the reactivity of the S_2-state towards endogenous agents is very big in the two systems. Thus, in *Oscillatoria* the S_2-state reacts with exogenous oxygen [3], whereas in tobacco chloroplasts it does not [3]. This reaction leads in *Oscillatoria* thylakoids to the production of free hydrogen peroxide which upon the successive absorption of two photons is decomposed to give oxygen and protons (and electrons). The *Oscillatoria* system thus seems accessible to oxygen or more reactive by principle. It should be noted that in higher plants H_2O_2 is electron donor to photosystem II under certain conditions of chloride and/or calcium deficiency [21, 22]. Clearly under the oxygen partial pressure of normal air, the water-oxidizing complex of *Oscillatoria* interacts with oxygen. Addition of hydrazine to the *Oscillatoria* thylakoids leads to an interaction of this compound with the hydrogen peroxide forming and decomposing reaction (Fig. 2). It appears that in *Oscillatoria* hydrazine is oxidized by photosystem II in an univalent oxidation reaction leading to dinitrogen evolution (Fig. 3). The mechanism of this oxidation reaction corresponds to the earlier described one in tobacco chloroplasts [13]. By applying equimolar quantities of $^{15}NH_2-{}^{15}NH_2$ and $^{14}NH_2-{}^{14}NH_2$ and measuring the observed masses of the nitrogen evolved it is clearly seen that in this oxidation reaction the N–N-bond is not split (Fig. 7). Oxidation should therefore, as described earlier, go *via* the $HN^{\circ}-NH_2$-radical [13]. As a difference to the tobacco chloroplast system dinitrogen evolution requires the water oxidase which is demonstrated by the fact that dinitrogen evolution is eliminated by Tris-washing (Figs. 1 and 8). Fig. 2 and 5 clearly show that hydrazine interacts with the hydrogen peroxide forming and decomposing system, which is to say that hydrazine preponderantly interacts with the S_2-state. The presented data shows that hydrazine oxidation deactivates the S-state system into a condition which is more reduced than S_0, thus leading to the two digit shift of a flash pattern reported in the literature [10] or the delayed oxygen evolution of a cumulated signal of a train of ten flashes as shown in Fig. 5.

Acknowledgements

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