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MASS SPECTROMETRIC STUDIES OF HYDRAZINE PHOTOOXIDATION BY ILLUMINATED CHLOROPLASTS

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Mass spectrometric techniques were used to monitor directly the products evolved during the course of hydrazine (NH_2NH_2) photooxidation by chloroplasts exposed to short saturating flashes or continuous high light. We found that: (1) Molecular N_2 was the sole volatile product of hydrazine photooxidation. Isotopic studies showed that the N-N bond remained intact during the $NH_2NH_2 \rightarrow N_2$ transformation. Under conditions in which spurious side reactions were minimized (see item 3 below), the N_2 yield was equal to the O_2 yield during H_2O photooxidation. (2) In the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, N_2 was evolved, but only on the first flash, suggesting that N_2 was formed by the combination of single-electron oxidation products of hydrazine. (3) In addition to its production by Photosystem II, N_2 can also be generated by a series of secondary reactions mediated by superoxide. This 'extra' N_2 evolution can be eliminated by the addition of superoxide dismutase. Our results indicate that hydrazine can be used as a reliable probe of Photosystem II provided that (a) N_2 evolution (rather than O_2 uptake) is monitored, and (b) precautions are taken to minimize spurious side reactions. Under conditions in which the participation of superoxide is minimized, N_2 evolution accurately reflects the photooxidation of hydrazine by Photosystem II.

Introduction

Hydrazine (NH_2NH_2) can serve as an electron donor to PS II and thus can be used to study reactions near the site of O_2 evolution [1,2]. At low concentrations, NH_2NH_2, like hydroxylamine (NH_2OH), can effect a delay in the normal, O_2 flash-yield pattern without significantly decreasing O_2-evolution capacity [3]. Thus, this compound is one of a rather small group of electron donors that can be oxidized by PS II and not destroy the O_2-evolving system [4].

Early hydrazine photooxidation studies [1,2] showed that the rate of hydrazine-supported O_2 uptake could significantly exceed the rate of PS II turnover. The excess O_2 uptake was attributed to the interaction of hydrazine or one of its oxidation products with dissolved O_2 [1].

In a previous report [5], we described the results of experiments in which we used mass spectrometric techniques to monitor directly the products generated during the photooxidation of hydroxylamine by isolated chloroplasts. We found that molecular N_2, formed by the combination of two one-electron oxidation products of NH_2OH, was the only stable reaction product.

In this communication, we describe experiments in which we studied the processes involved during the photooxidation of hydrazine by PS II. We found that, as in the case of hydroxylamine, molecular N_2 was the only significant stable reaction product. However, unlike hydroxylamine, hydrazine can also be oxidized via a series of secondary reactions, which also produce N_2. Our data were consistent with a reaction scheme in which the interaction of NH_2NH_2 and O_2^-

Abbreviations: PS, Photosystem; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)-methylglycine.

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serves to generate the quantities of N_2 above and beyond that attributable to the direct oxidation of NH_2NH_2 by PS II. In agreement with this scheme, we found that the stoichiometry between PS II-generated oxidant and N_2 could be varied widely by altering the concentration of internally generated O_2^-.

Materials and Methods

The mass spectrometric apparatus and techniques for directly monitoring the gas exchange elicited by short saturating flashes and continuous light were described earlier. In the flashing-light experiments, the chloroplast suspension was carefully layered on a semipermeable membrane that served as the mass spectrometer inlet [5,10]. This configuration afforded good sensitivity (pmol/flash) and a time response of less than 1 s. In the continuous-light experiments, a stirred illuminated sample was monitored using a semipermeable membrane inlet [6,7].

Fluorescence measurements were done using procedures similar to those of Doschek and Kok [18]. The results of each row of Table I were obtained from a fluorescence-rise curve under the given conditions. A 4 min dark period preceded each sequence. Each group of five observations was done, in the order shown, on the same sample.

Tris-extracted chloroplasts were used for all experiments except those in Table I. Chloroplasts were isolated [8] from greenhouse or market spinach in medium containing 0.4 M sucrose, 0.01 M NaCl and 0.05 M Tris-HCl (pH 7.4). These chloroplasts were extracted by diluting a 1–2 ml aliquot (approx. 4 mg Chl/ml) to 100 ml with a high-Tris solution (0.4 M sucrose, 0.01 M NaCl, 0.75 M Tris-HCl, pH 8.1) and stirring this suspension for 20 min in a cold room under dim light [9]. The chloroplasts were then centrifuged, washed and resuspended in the isolation medium. The extraction efficiency, based on the extent of inhibition of the Hill reaction, was greater than 95%.

[^{15}N]Hydrazine sulfate (95 atom% ^{15}N) was obtained from Prochem, London, U.K.

Results and Discussion

Products of hydrazine photooxidation

Fig. 1 shows the relative flash yields – at *m/e* values corresponding to likely oxidation products – when chloroplasts were subjected to a train of saturating flashes (3-s spacing) in the presence of 1 mM NH_2NH_2. Note that molecular N_2 was the only volatile stable oxidation product formed in significant amounts; there was no apparent evolution of nitrogen oxides. Similar results were obtained in continuous light: only *m/e* values corresponding to molecular N_2 increased when chloroplasts were subjected to strong continuous light in the presence of 10 mM NH_2NH_2

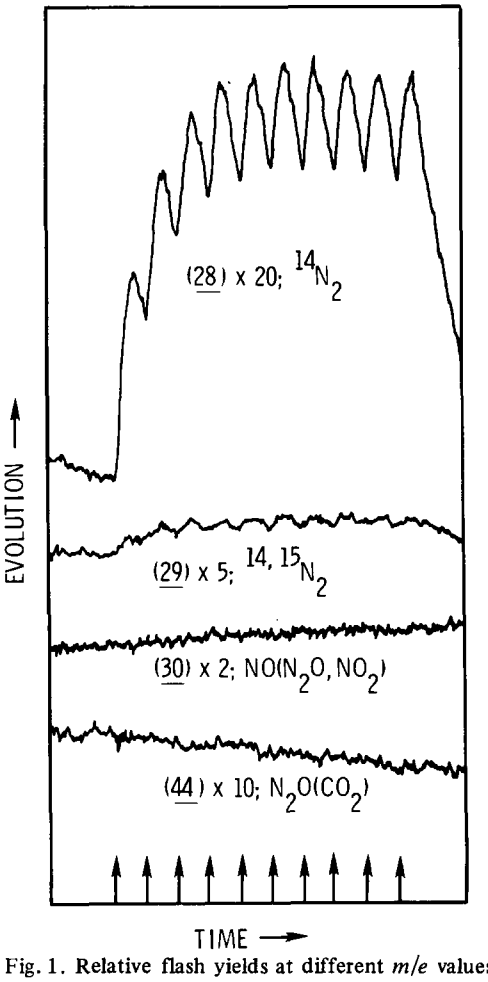

Fig. 1. Relative flash yields at different *m/e* values observed when 10 μl of Tris-extracted chloroplasts (5.8 Chl/ml) were subjected to a series of flashes (arrows) in the presence of 1 mM NH_2NH_2. Numbers given after the *m/e* values refer to different attenuations; e.g., *m/e* = 28 was attenuated 10-fold compared to *m/e* = 30. The different traces were sequentially obtained in various orders after a dark time of 5 min. See text and Ref. 5 for other details.

and saturating amounts of ferredoxin and NADP.

We specifically searched for the production of NH_3 (which can be produced during the oxidation of NH_2NH_2 to N_2 or NH_3 [11]) by running a similar series of experiments both at pH 7.4 and at pH values 9.0 and 9.5, near the pK_a of the NH_3-NH_4^+ couple. In these experiments, we used a solid CO_2/acetone trap to remove most of the H_2O, thus greatly facilitating the sensitivity of detection at $m/e = 17$ (NH_3). We found no evidence of NH_3 evolution during the NH_2NH_2 photooxidation process; any putative NH_3 production would be less than 1% of the observed N_2 production. The absence of significant NH_3 production under these conditions also seems to rule out the production of azide (N_3^-), a nonvolatile compound not detectable with our experimental technique, since azide production is accompanied by NH_3 production during the NH_2NH_2 oxidation reaction [11].

TABLE I

INITIAL FLUORESCENCE YIELD AFTER A SATURATING FLASH WITH DIFFERENT DONORS IN THE PRESENCE AND ABSENCE OF DCMU

The tabulated fluorescence was calculated from the expression, $(F_i - F_{i,min})/(F_m - F_{i,min})$, where F_i = initial fluorescence level under the stated conditions, F_i,min = initial fluorescence level in dark-adapted samples in the absence of DCMU, and F_m = maximum fluorescence level attained after prolonged illumination (>10 s). $F_{i,min}$ was identical in the three cases; F_m values varied by about 10%. The reaction mixture for each experiment contained 50 mM Tricine (pH 7.4), 5 mM $MgCl_2$, and chloroplasts (6 μg Chl/ml) in addition to the reagents given in the table. The tabulated values for each of the three cases were obtained in the order shown using the same sample of unextracted, O_2-evolving, chloroplasts. Similar results with NH_2OH and NH_2NH_2 were obtained with Tris-extracted chloroplasts.

	Normalized initial fluorescence
No added donor (H_2O oxidation)	0
Flash + 0.1 s dark	0.07
DCMU (10^{-5} M)	0.13
Flash + 2 s dark (+DCMU)	0.69
Flash + 10 s dark (+DCMU)	0.27
NH_2OH (2 mM)	0
Flash + 0.1 s dark	0.06
DCMU (10^{-5} M)	0.41
Flash + 10 s dark (+DCMU)	0.77
Flash + 5 min dark (+DCMU)	0.50
NH_2NH_2 (5 mM)	0
Flash + 0.1 s dark	0.08
DCMU (10^{-5} M)	0.40
Flash + 10 s dark (+DCMU)	0.74
Flash + 5 min dark (+DCMU)	0.50

Fig. 2 shows the results obtained when we ran the same experiment in the presence of ^{15}N-labeled substrate. As shown in the top traces, the major product appeared at $m/e = 30$, the spectral position of $^{15}N_2$, when the reaction was run with 95 atom% ^{15}N hydrazine. A small amount of $^{14,15}N_2$ at $m/e = 29$, consistent with the isotopic composition of the substrate, was also evolved.

The bottom traces of Fig. 2 show the results obtained when the same experiment was run in the presence of equimolar amounts of labeled and unlabeled substrate. These data suggest that little, if

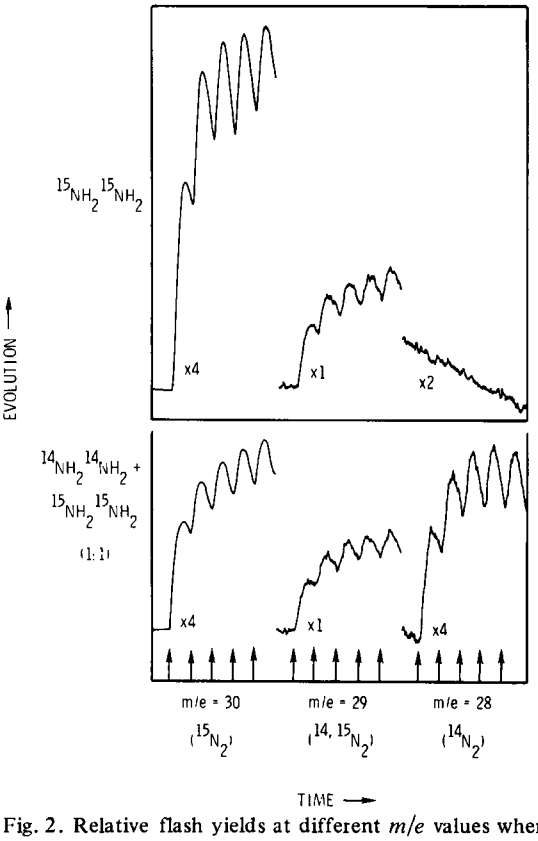

Fig. 2. Relative flash yields at different m/e values when 10 μl of Tris-extracted chloroplasts (6.1 mg Chl/ml) were subjected to a series of flashes in the presence of 1 mM $^{15}NH_2{}^{15}NH_2$ (top) or an equivalent mixture (500 μM each) of labeled and unlabeled NH_2NH_2 (bottom).

any, N interchange, manifest as isotopic scrambling, occurs during the production of molecular dinitrogen from hydrazine. If the nitrogen isotopes were to be completely randomized during the series of reactions leading to N_2, one would predict that the $^{15}N_2$: $^{14,15}N_2$: $^{14}N_2$ ratio would be 0.23 : 0.50 : 0.27. In reality, quite the opposite was observed; the data of Fig. 2 * indicate that less than 3% of the N atoms interchanged, suggesting that the N-N bond remains intact during the conversion of NH_2NH_2 to N_2.

The fact that N_2 was evolved on the first flash in all of these experiments indicates that, like NH_2OH, the oxidation of NH_2NH_2 involves the production of a one-electron photooxidation product. Subsequent (nonphotochemical) interactions lead to the evolution of molecular N_2.

Effect of DCMU

Table I is a compilation of the initial fluorescence yields observed after a saturating flash with different donors (H_2O, NH_2OH and NH_2NH_2) in the presence and absence of DCMU. These results show that, in the presence of DCMU, NH_2NH_2, like NH_2OH (see also Ref. 12), prolongs the period of high fluorescence yield following a saturating flash. This, in turn, suggests that NH_2NH_2, like NH_2OH, inhibits the back reaction between $P\text{-}680^+$ (the oxidized PS II donor) and Q^- (the reduced primary acceptor) and that the reduction of $P\text{-}680^+$ by NH_2NH_2 is irreversible, in contrast to the reduction of $P\text{-}680^+$ by the O_2 system [13].

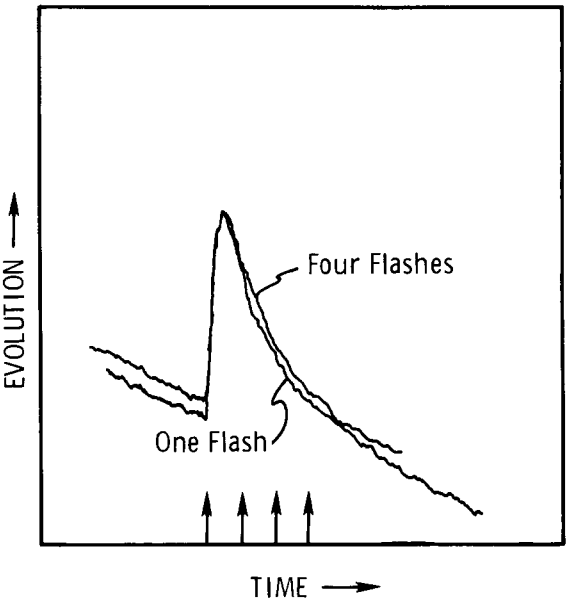

Fig. 3. Relative yields of N_2 elicited by one flash and four flashes (3-s spacing) in the presence of NH_2NH_2 (1 mM) and DCMU (10^{-4} M).

Fig. 3 shows a comparison of the N_2-evolution pattern elicited by one flash and four flashes in the presence of DCMU (added in complete darkness). A comparison of the two traces indicates that N_2 was evolved, but only on the first flash. A similar comparative experiment with NH_2OH yielded virtually identical results (data not shown), corroborating an earlier report [5]. This indicates that, like NH_2OH, the oxidation of NH_2NH_2 is strictly a PS II reaction under these conditions.

The irreversibility of the NH_2NH_2-DCMU system, like that of the NH_2OH-DCMU system, can thus be attributed to the ability of these donor systems to discharge immediately oxidizing equivalents as N_2. This contrasts with the normal DCMU-inhibited system, in which the oxidizing equivalents are retained (primarily as S_2) and can subsequently back react with Q^-.

Stoichiometry and the involvement of O_2

Table II summarizes the results of a series of experiments in which the flash yields of N_2 evolution and O_2 uptake were determined as a function of acceptor and O_2 tension using NH_2NH_2 and NH_2OH

* We routinely observe that the 29/28 ratio of the N_2 evolved during oxidation of unlabeled NH_2NH_2 or NH_2OH is higher, by a factor of about 2, than one would predict from normal isotopic abundance. This problem can be corrected, albeit with a substantial loss of time response and signal-to-noise ratio, by interposing a solid CO_2/acetone trap between the inlet and the mass spectrometer ionizer. This spurious signal probably reflects the protonation of N_2 within the mass spectrometer; i.e., $N_2 + H^+ \rightarrow N_2H^+$ analogous to the well-known reaction:

$$H_2O \rightarrow OH + H^+$$

$$H_2O + H^+ \rightarrow H_3O^+ (m/e = 19)$$

When ^{15}N-labeled NH_2NH_2 is photooxidized, a comparable H_2O-dependent signal is observed at $m/e = 31$ ($^{15}N_2H^+$).

TABLE II

N_2 AND O_2 FLASH YIELDS AS A FUNCTION OF ACCEPTOR AND O_2 TENSION

Tabulated values refer to relative peak height of the first flash after 5 min dark; the N_2 and O_2 flash yields are not directly comparable. Subsequent flash yields with NH_2OH did not change significantly (see Ref. 5); those with NH_2NH_2 decreased 10–20% by the tenth flash. O_2 levels: high, 32–47% (430–630 μM); low, 1.8–2.0% (24–27 μM). Acceptor concentrations: 0.4 mM NADP plus 20 μg ferredoxin plus 1 mM $MgCl_2$; Methyl viologen (MV): 10^{-4} M.

$[O_2]$	Acceptor	Hydrazine		Hydroxylamine	
		N_2 evolution	O_2 uptake	N_2 evolution	O_2 uptake
Low	None	23	14	48	4
High	None	46	52	40	11
Low	NADP	21	10	42	1
High	NADP	33	36	40	2
Low	MV	18	11	42	5
High	MV	40	59	47	35

as donors. Several salient points can be gleaned from these data:

(1) At low O_2 tensions, the N_2 yield with NH_2NH_2 was about one-half that observed with NH_2OH. Since with NH_2OH the yield is $\frac{1}{2}$ N_2/trap per flash [5], the data suggest that $\frac{1}{4}$ N_2/trap per flash was evolved under low-O_2 conditions.

(2) At high O_2 tensions, the NH_2NH_2 N_2 flash yields were approximately double those observed at the low conditions, whereas the NH_2OH N_2 yields were unaffected by O_2 tension. This suggests that N_2 is formed by either the reaction of molecular O_2 with an NH_2NH_2 oxidation product or NH_2NH_2 with an O_2 reduction product.

(3) O_2 uptake is strongly correlated with the production of N_2 from NH_2NH_2 (but not from NH_2OH). The (approximately) 2-fold difference in N_2 evolution between low-O_2 and high-O_2 conditions is associated with large differences in O_2 uptake in all three cases.

Taken together, the data of Table II point to the participation of O_2 (or its reduction products) in some of the reactions leading to the production of N_2 from NH_2NH_2. These secondary reactions probably underlie the unusual stoichiometry of these data (see also Ref. 1).

Hydrazine oxidation under high-light conditions

Figs. 4 and 5 show the results obtained when N_2 evolution and O_2 uptake were monitored during hydrazine photooxidation under various conditions in continuous high light. These data, obtained using either ferredoxin-NADP or methyl viologen as the terminal electron acceptor, show the rates as a function of NH_2NH_2 concentration under high-O_2 conditions (greater than 1 mM, Figs. 4A and 5A) and as a function of O_2 tension (Figs. 4B and 5B). Experiments were done both in the presence and absence of saturating amounts of superoxide dismutase.

One of the most striking aspects of these data is the large effect of superoxide dismutase. In all cases (except zero O_2) the addition of this enzyme resulted in a large decrease in N_2 evolution and O_2 uptake. Superoxide dismutase also dramatically affected the shape of the substrate saturation curve. As shown in Figs. 4A and 5A, the rates of N_2 evolution and O_2 uptake saturated at approx. 30 mM NH_2NH_2 with both the ferredoxin-NADP and methyl viologen acceptor systems in the presence of superoxide dismutase; in the absence of added enzyme there was no apparent substrate saturation up to 100 mM. These data suggest that superoxide can play a large role in N_2 evolution as well as O_2 uptake during hydrazine photooxidation. The addition of superoxide dismutase seems to control side reactions so that N_2 evolution accurately reflects PS II activity.

The results obtained in the presence of superoxide dismutase can be satisfactorily explained by the fol-

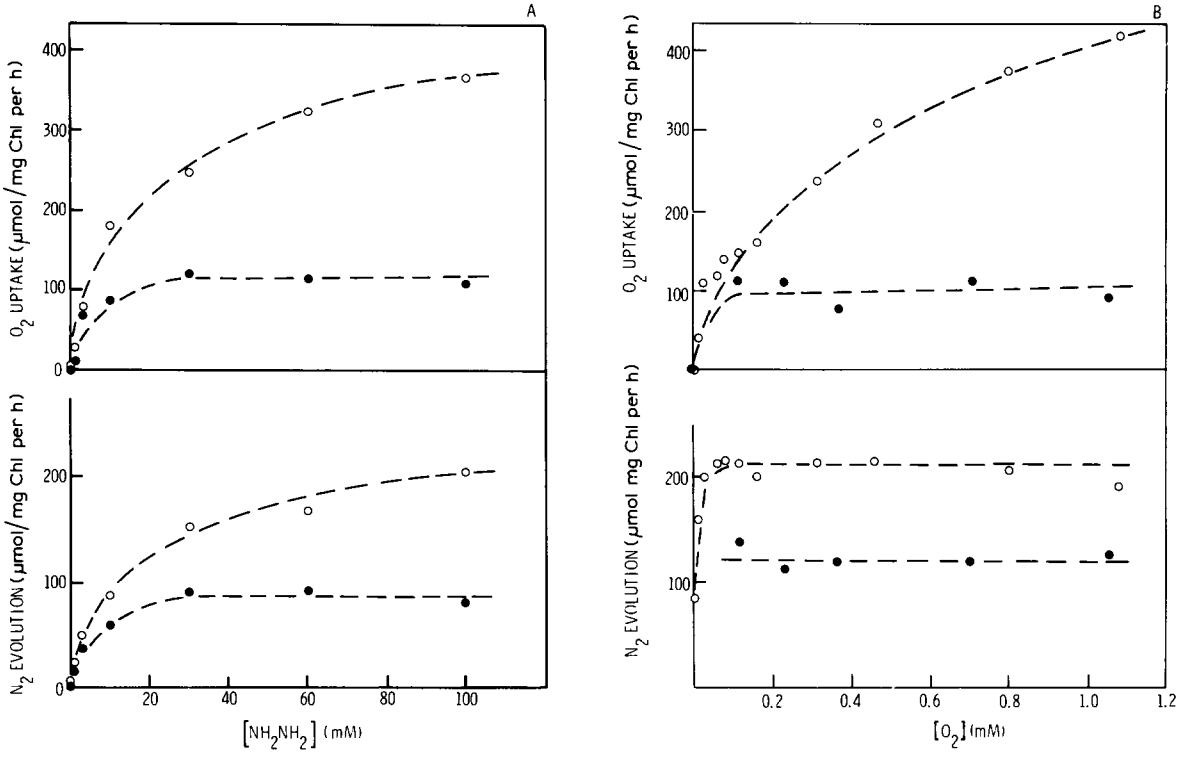

Fig. 4. Rates of N_2 evolution and O_2 uptake in continuous high light as a function of NH_2NH_2 concentration and O_2 concentration with ferredoxin-NADP as the electron acceptor. The experiments of A were all done at high O_2 (greater than 1 mM); those of B were all done using 30 mM NH_2NH_2. In addition to substrate and O_2, the reaction medium contained 50 mM Tricine (pH 7.4), 30 mM methylamine, 1 mM $MgCl_2$, and 500 U catalase. Acceptor system: 40 μg ferredoxin/ml, 2 mM NADP. Chloroplast concentration was equivalent to 50 μg Chl/ml. Saturating amounts of superoxide dismutase (1200 U/ml, determined using methyl viologen, see legend of Fig. 5) were added as noted in figure. ○, superoxide dismutase absent; ●, superoxide dismutase present.

lowing set of reactions:

$$NH_2NH_2 \xrightarrow{PSII} NH_2NH^{\cdot} \quad (1)$$

$$2\,NH_2NH^{\cdot} \rightarrow NH{=}NH + NH_2NH_2 \quad (2)$$

$$2\,NH{=}NH \rightarrow N_2 + NH_2NH_2 \quad (3)$$

$$O_2 \xrightarrow{PSI} O_2^- \quad (4)$$

$$2\,O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

Eqns. 1–3 illustrate the (possibly idealized) production of molecular N_2 via the one-electron oxidation product of NH_2NH_2. These reactions, probably predominate under conditions of low NH_2NH_2, low O_2, and low light (Figs. 1 and 2, low-O_2 point in Fig. 4B). The participation of diimine (NH=NH) in this reaction sequence is consistent with earlier reports [11]. Diimine can be produced by the oxidation of NH_2NH_2; in the absence of other unsaturated species (e.g., olefins) it disproportionates.

Eqns. 4 and 5 illustrate the generation of superoxide (O_2^-) by PS I (see, for example, Ref. 14) and its breakdown by dismutation. The latter reaction can be greatly accelerated by the addition of superoxide dismutase. The production of O_2^- by PS I is strongly dependent upon the terminal electron acceptor. In the presence of methyl viologen, O_2^- is efficiently generated at very high rates (equal to those set by limitations in electron transport) at rather modest O_2 tensions [15]. It also formed, albeit at somewhat lower rates, in the presence of ferredoxin [14]. This reaction probably underlies the somewhat anomalous results shown in Fig. 4B, which suggest that at O_2

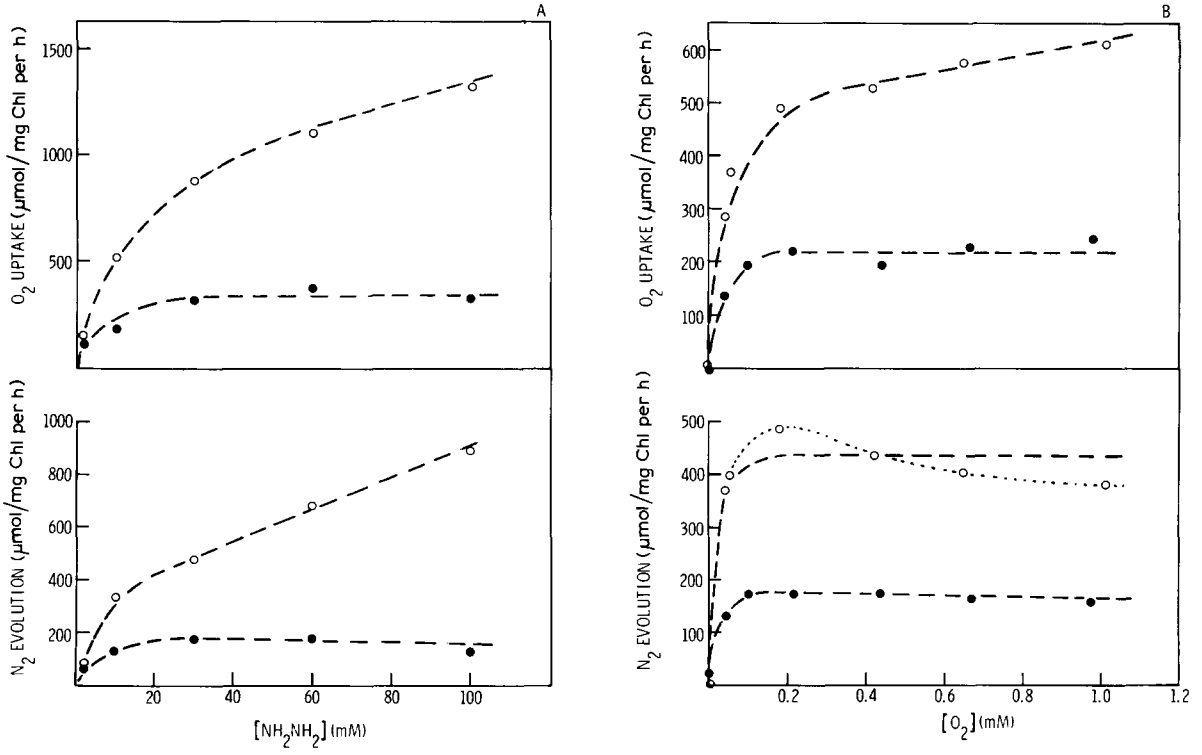

Fig. 5. Rates of N_2 evolution and O_2 uptake in continuous high light as a function of NH_2NH_2 concentration and O_2 concentration with methyl viologen (10^{-4} M) as acceptor. See legend of Fig. 4 for details. The dotted line in B illustrates an alternative interpretation of the data. The existence and significance of this apparent maximum were not studied in detail. ○, superoxide dismutase absent; ●, superoxide dismutase present.

tensions not much removed from zero the reduction of O_2 competes very favorably with that of NADP under the chosen reaction conditions.

In the presence of superoxide dismutase, N_2 evolution depends on O_2 only to provide a terminal electron acceptor (via methyl viologen or ferredoxin). The O_2 saturation kinetics of this PS I-mediated reaction are shown for the case of methyl viologen by the rapid rise of N_2 evolution in Fig. 5B (+SOD curve). The equivalent N_2 curve for the ferredoxin-NADP case (Fig. 4B) is masked by the apparent switch of ferredoxin oxidation from NADP to O_2 at rather low O_2 tensions.

The PS II-mediated level of N_2 evolution with saturating NH_2NH_2 in the presence of superoxide dismutase is about 100 μmol/mg Chl per h with ferredoxin-NADP and about 175–200 μmol/mg Chl per h with methyl viologen. These differences probably reflect disparities in the efficiency of the two acceptor systems under the chosen assay conditions. In the presence of superoxide dismutase, the N_2 evolution/O_2 uptake stoichiometry was near unity in all cases in accordance with the reaction scheme:

$$4\ NH_2NH_2 \xrightarrow{PSII} N_2 + 3\ NH_2NH_2 + 4e^- \text{ (Eqns. 1–3)}$$

$$4e^- + 4\ O_2 \xrightarrow{PSI} 4\ O_2^-$$

$$4\ O_2^- + 4\ H^+ \xrightarrow{\text{superoxide dismutase, catalase}} 3\ O_2 + 2\ H_2O$$

In the absence of superoxide dismutase, the observed reactions are much more difficult to explain. Eqns. 6 and 7 illustrate two possibly relevant secondary reactions by which N_2 production (and O_2 uptake) could be enhanced (see Refs. 16 and 17):

$$NH_2NH^{\cdot} + O_2 \rightarrow NH = NH + O_2^- + H^+ \quad (6)$$

$$NH_2NH_2 + O_2^- + H^+ \rightarrow H_2O_2 + NH_2NH^{\cdot} \quad (7)$$

Eqn. 6 describes the attack of O_2 by the hydrazyl (oxidized hydrazine) radical, a reaction which competes with disproportion (Eqn. 2). Eqn. 7 describes the attack of NH_2NH_2 by O_2^-. Since this reaction competes with the reaction of Eqn. 5 for O_2^-, it can be effectively eliminated by the addition of large amounts of superoxide dismutase. Note that if both these reactions were to proceed rapidly compared to the chain-termination reactions (Eqns. 2 and 5), they would form the basis of an autocatalytic chain-reaction sequence leading to the production of large amounts of N_2 per PS II turnover.

The data of Figs. 4 and 5, in particular the effect of superoxide dismutase, indicate that the oxidation of NH_2NH_2 by O_2^- makes a substantial contribution to N_2 evolution (and O_2 uptake) in the absence of added enzyme. The observed N_2-evolution rates with ferredoxin-NADP are probably consistent with a scheme in which equal amounts of N_2 are generated by PS II (Eqns. 1–3) and PS I (via Eqns. 4, 7, and 1–3). A similar mechanism may also predominate with methyl viologen. The lack of any significant increase of N_2 evolution with increasing O_2 concentration suggests that the attack of O_2 by the hydrazyl radical (Eqn. 6) does not make a substantial contribution to N_2 evolution in these experiments.

We have no ready explanation for the anomalously high rate of O_2 uptake observed in the absence of superoxide dismutase, particularly at high NH_2NH_2 and O_2 concentrations (Fig. 5A). These observations may reflect the participation of O_2^- in additional side (dark) reactions that result in the oxidation of NH_2NH_2 and the production of nonvolatile products rather than molecular N_2 [11].

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