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METABOLISM OF ELEMENTAL SULPHUR AND OXIDATION OF SULPHITE BY WHEAT AND SPINACH CHLOROPLASTS

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; *Triticum aestivum*; Gramineae; chloroplasts; ^{35}S; elemental S; S compounds; sulphite oxidase.

Abstract—Wheat or spinach chloroplasts were fed with ^{35}S-labelled elemental sulphur (S^0). ^{35}S-labelled compounds appearing thereafter were analysed. Five ^{35}S-compounds were recovered, thiosulphate, sulphite, sulphate, cysteine and glutathione. The effect of environmental conditions was also studied. The measurement of S^0 metabolism rate was compared to the recovery of the introduced S^0 into other S compounds. The specific radioactivity of thiosulphate and sulphate was determined. The results obtained have permitted the elucidation of the precise biochemical pathway of S^0 oxidation. The oxidation of sulphite in wheat and spinach chloroplasts occurs via both non-enzymatic (in particular the operation of the photosynthetic electron transport chain) and enzymatic processes (sulphite oxidase activity).

INTRODUCTION

When elemental sulphur (S^0) is applied to wheat leaves, a small proportion (1.6–2%) is absorbed and metabolized into sulphate, S-amino acids and proteins [1, 2], suggesting a direct oxidation of S^0. It has been established recently that $^{35}S^0$ is oxidized to $^{35}SO_4^{2-}$ by fresh, but not by boiled wheat chloroplasts [3–5]. The oxidation process is slow but generates $S_2O_3^{2-}$ and SO_3^{2-}. Enzymatic systems involved in primary S^0 oxidation to thiosulphate or sulphite have not been reported for higher plants, but have been purified from bacteria. On the other hand, two pathways have been described for the metabolism of sulphite inside chloroplasts. One possibility is the direct reduction of sulphite into highly toxic hydrogen sulphide which may be metabolized into cysteine or released in part into the atmosphere [6, 7]. Sulphite reductase is a thylakoid-bound enzyme and synthesis of cysteine requires a stromal enzyme [6, 8]. The other pathway is the oxidation of sulphite to sulphate, proceeding via a radical chain reaction involving light-dependent photosynthetic electron transport [7, 9]. Oxidation of Na_2SO_3 into SO_4^{2-} was observed in chloroplasts incubated in either the light or dark [4, 10]. Therefore, processes in addition to the non-enzymatic aerobic oxidation must be occurring. In the present report, we confirm the oxidation of S^0 in spinach chloroplasts and investigate the nature of the S compounds synthesized from S^0 using ^{35}S-labelling. The oxidation of sulphite was elucidated in the absence of the radical-dependent oxidation chain reaction. The pathways of S metabolism were compared in wheat and spinach chloroplasts.

RESULTS AND DISCUSSION

Metabolism rate of $^{35}S^0$

The determination of S^0 metabolism was studied with the addition of $^{35}S^0$ to intact chloroplasts isolated from wheat or spinach leaves. At the end of experiments, the lamellar membranes were pelleted by centrifugation and the radioactivity was determined in the supernatant. In the case of boiled chloroplasts, a small amount of label was present in the supernatant fraction which corresponded to a low level of solubilized S^0 under the experimental conditions. Supernatant radioactivity was higher in the case of fresh chloroplasts. The difference between supernatant labelling in fresh and boiled chloroplasts was a measure of the transformation of $^{35}S^0$ into soluble ^{35}S-compounds by chloroplastic processes [5]. This S^0 transformation was expressed as a percentage of the initial radioactivity offered to chloroplasts. The results obtained from experiments carried out for 2 hr with spinach chloroplast suspensions (0.1 mg chlorophyll ml^{-1}) were similar to results obtained previously with wheat chloroplasts under the same conditions (Table 1). In the light, between 3.5 and 8.2% of the $^{35}S^0$ was transformed into soluble S compounds by chloroplasts (1–4 μ atom S mg^{-1} chlorophyll). $^{35}S^0$ metabolism was decreased in the dark but remained significant (1–4.5% of initial $^{35}S^0$, i.e. 0.2–2 μ atom S mg^{-1} chlorophyll).

Analysis of S *compounds produced*

Because it was observed that S^0 was transformed into soluble products by chloroplasts, the nature of these S

Table 1. $^{35}S^0$ metabolism by spinach and wheat chloroplasts (0.1 mg chlorophyll ml^{-1}) during 2 hr: percentage of initial radioactivity recovered as soluble S compounds and corresponding S^0 transformation

Chloroplasts	Initial S^0 concentration (μ atom ml^{-1})	S compounds radioactivity (% of initial RA) Light	Dark	S^0 transformation (μ atom mg^{-1} chl) Light	Dark
Spinach*	2	5.3 ± 1.7	4.5 ± 4.5	1.1 ± 0.3	0.9 ± 0.9
	5	4.9 ± 2.5	1.0 ± 0.1	2.4 ± 1.2	0.5 ± 0.1
Wheat†	2	8.2 ± 6.5	1.0	1.7 ± 1.3	0.2
	11	3.5 ± 0.4	1.8 ± 1.1	3.9 ± 0.4	2.0 ± 1.2

*Means ± s.d. of three different chloroplast preparations.
†Results from ref. [5].

compounds was investigated. A portion of S^0 metabolized was found to be SO_4^{2-}, as proved by $BaCl_2$ precipitation. In light experiments, 13–46% of $^{35}S^0$ metabolized consisted of $^{35}SO_4^{2-}$. Sulphate was determined concurrently by the use of the turbidimetric method described in the Experimental. The level of sulphate was obtained by the two methods with good agreement, but the turbidimetric method had a lower reproducibility with a relative error increased by 25%.

The conversion of S^0 to other S compounds was investigated by HPLC after transformation to monobromobimane derivatives. Thiosulphate, sulphite, cysteine and glutathione were detected by fluorimetry. Their amounts were determined with the help of standards and their ^{35}S-labelling was measured using an in-line flow-through radioactivity monitor. The presence of $^{35}SO_4^{2-}$ was detected as a radioactive peak at the beginning of the radiochromatograms. The levels of endogenous pools of thiosulphate, sulphite and sulphate were low when spinach and wheat chloroplasts were maintained without S^0 (Table 2). After 2 hr in the presence of a high S^0 concentration (5 or 11 μatom S ml^{-1}), the pools of $S_2O_3^{2-}$ and SO_4^{2-} increased, with that of SO_3^{2-} increasing at a lower rate. On the contrary, in consideration of the precision of the data, no significant variation of cysteine and glutathione pools was observed. These results showed that S^0 would be metabolized preferentially in chloroplasts through an oxidation process. Under N_2, S^0 oxidation can be measured in the light with photosynthetic O_2 and in the dark with residual O_2 in the chloroplast suspension and reaction medium [4]. There was always more thiosulphate and sulphate in wheat chloroplasts than in those of spinach. In wheat, $S_2O_3^{2-}$ and SO_3^{2-} pool levels were lower under aerobic conditions than under anaerobic ones, indicating an easier oxidation of S^0 into sulphate under O_2. As expected, a higher sulphate pool

Table 2. S compounds production (nmol mg^{-1} chlorophyll) and their respective radioactive labelling ($10^3 \times$ counts) in brackets, observed in wheat or spinach chloroplasts maintained for 2 hr under different light and atmosphere conditions in the presence of $^{35}S^0$ (initial amount: 5 or 11 μ atom ml^{-1}) or without S^0. Data are the mean of two different chloroplast preparations (relative error 30%).

	Wheat					Spinach				
	Without S^0	Oxygen		Nitrogen		Without S^0	Oxygen		Nitrogen	
S compound		Light	Dark	Light	Dark		Light	Dark	Light	Dark
$S_2O_3^{2-}$	6.9	336*	350*	922*	380*	12.0	90	144	546	86
		(1626)	(1782)	(4718)	(2520)		(340)	(708)	(5708)	(842)
SO_3^{2-}	4.1	6.0*	15.0*	15.4*	18.0*	0	19.6	19.2	26.2	14.6
		(350)	(nd)	(274)	(136)		(nd)	(nd)	(70)	(64)
SO_4^{2-}	12.4	2068*	1190*	1652*	774*	0	116	152	472	38
		(1264)	(664)	(838)	(392)		(248)	(770)	(1392)	(nd)
Cysteine	9.0	12.0	15.8	15.2	26.4	24.4	21.8	30.8	20.8	22.4
		(322)	(314)	(474)	(136)		(105)	(74)	(nd)	(32)
Glutathione	1.3	0	2.6	7.0	17.6	18.3	16.4	24.2	18.8	0
		(nd)	(240)	(256)	(6)		(248)	(nd)	(526)	(nd)

*Results from ref. [5].
nd: not detectable.

was observed concurrently under O_2. In the case of spinach chloroplasts, S^0 oxidation seemed to be low under N_2 in the dark, probably due to a lack of enough residual O_2. In the light, a decrease of thiosulphate and sulphite levels was observed under O_2 with respect to experiments carried out under N_2, as found in wheat chloroplasts, but unexpectedly no more sulphate was obtained. It has been noted that the size of the sulphite pool is relatively low in spinach chloroplasts, as well as in those of wheat. As previously reported [5], three hypotheses could be proposed to explain this fact, (i) a fast turnover of sulphite, (ii) a slow transformation of thiosulphate to sulphite leading to a thiosulphate accumulation and (iii) production of thiosulphate and sulphite from S^0 by two different pathways not closely related, with a weaker importance of sulphite synthesis. As cysteine and glutathione pools were virtually constant whatever the light and atmosphere conditions, it was not possible to determine if these two compounds were implicated in S^0 metabolism. At the very least, the presence of S^0 did not lead to their accumulation. A study of ^{35}S-labelling would be very useful in understanding the biochemical pathways of S metabolism.

All S compounds considered were ^{35}S-labelled (Table 2). The addition of $^{35}S^0$ to standard solutions of thiosulphate, sulphite or sulphate at the same pH, temperature and time conditions as those used in chloroplast experiments did not lead to spontaneous labelling of S compounds. The observed labelling of S compounds in chloroplast suspensions could not be due to an isotopic exchange between $^{35}S^0$ and pre-existent endogenous S compounds; there is no doubt that thiosulphate, sulphite, sulphate, cysteine and glutathione were synthesized from $^{35}S^0$. $S_2O_3^{2-}$ was extensively labelled, presumably because of doubly labelling. For SO_3^{2-}, cysteine and glutathione, the detection threshold was not always reached and their labelling was not systematically studied. However, the labelling of cysteine and glutathione proves that these reduced compounds are implicated in S^0 metabolism. S-containing amino acids are classically synthesized in chloroplasts via the assimilatory sulphate reduction pathway [11]. Another pathway proposed is the direct reduction of free sulphite by sulphite reductase and the transformation of the intermediate hydrogen sulphide into cysteine [6, 7]. These two pathways are ferredoxin-dependent reactions and then light-dependent. It appears that the glutathione and cysteine pools are slightly more labelled in the light. It was reported that reduction of sulphate is far slower than reduction of sulphite [6, 7], but the results of ^{35}S-labelling presented here did not enable the distinction between the two possibilities.

Taking into account the efficiency of the radioactivity monitor used, the labelling of SO_4^{2-} and $S_2O_3^{2-}$ was converted into kBq and related to S amount (μg S) contained in sulphate and thiosulphate pools. These specific radioactivities were compared with the specific radioactivity of the $^{35}S^0$ offered to the chloroplasts (Table 3). The results showed that thiosulphate and sulphite could have been produced from $^{35}S^0$, since their specific radioactivity was lower than that of S^0. Except for sulphate in wheat chloroplasts, the specific radioactivity of thiosulphate and sulphate was in the same range as that of $^{35}S^0$ (maximal decrease of 40%). This shows that the biosynthesis pathway of thiosulphate and sulphate from S^0 was relatively straight and that there was little dilution with endogenous pools or from other biosynthetic pathways. The free sulphite, cysteine and glutathione pools analysed were very low, near to the detection threshold and labelling was not always observed. As a consequence, determination of their specific radioactivities was imprecise. However, it appeared that the specific radioactivities of sulphite (6.5 ± 2.3) and cysteine (5.2 ± 3.2) from spinach chloroplasts were compatible with the oxidation of S^0 successively through thiosulphate, sulphite and sulphate. Cysteine could be synthesized either from sulphate reduction or from sulphite reduction. On the other hand, in wheat chloroplasts, specific radioactivities of sulphite and cysteine appeared in a first analysis very high and must be verified (data not shown). Cysteine could be produced preferentially from direct sulphite reduction. This fact could explain the relatively high sulphate pool observed in wheat chloroplasts, since it would not be used in the assimilatory reduction pathway.

Table 3. Specific radioactivity of original $^{35}S^0$ and of thiosulphate and sulphate produced by chloroplasts

	Specific radioactivity (kBq μg^{-1} S)*		
	S^0	$S_2O_3^{2-}$	SO_4^{2-}
Wheat	4.8	4.9 ± 0.7	1.1 ± 0.1
Spinach	11.1	6.6 ± 3.1	7.8 ± 3.1

*Specific radioactivity for $^{35}S^0$ as provided by the supplier. Results for $S_2O_3^{2-}$ and SO_4^{2-} corresponded to mean ($\pm$ s.d.) for all experiments whatever experimental conditions.

It was possible to calculate the total S amount involved in the five S compounds analysed taking into consideration that two S atoms are present in thiosulphate and one S atom in the other compounds, sulphate, sulphite, cysteine and glutathione. This amount of recovered S was compared with the total S^0 amount consumed by the chloroplastic process estimated as described above (Table 4). It was observed that the metabolized S^0 was recovered in the S compounds analysed with a good yield under N_2 or in the dark. Lower recovery yields were observed in the light. The reductive pathway of sulphate which is a light-dependent process may have settled and could have led to S^0 incorporation into S compounds not detected under our experimental conditions, viz., bound-S compounds and proteins [12, 13]. On the other hand, the loss in recovery of S compounds could also have been due to the formation of volatile reduced compounds as mentioned by Dittrich *et al.* [7].

Sulphite oxidation by chloroplasts

The results of sulphite oxidation were expressed either as a percentage of sulphite transformed into sulphate

Table 4. Total S^0 transformation (initial amount, 5 or 11 μ atom ml^{-1}) by wheat or spinach chloroplasts for 2 hr and S recovery into S compounds analysed at the end of experiments. Data are the mean of two experiments

	Wheat*				Spinach			
	Oxygen		Nitrogen		Oxygen		Nitrogen	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
S^0 transformation† (μatom mg^{-1} Chl)	4.4	2.53	3.52	1.65	0.80	0.75	2.95	0.25
S recovery‡ (μatom mg^{-1} Chl)	2.76	1.92	3.53	1.60	0.35	0.51	1.63	0.25
Yield (%)	62.7	75.9	100	96.9	43.7	68.0	55.2	100

*Results from ref. [5].
†S^0 transformation was estimated by determination of soluble radioactivity as described in Table 1.
‡Total S recovered in sulphate, sulphite, thiosulphate, cysteine and glutathione (Table 2).

Table 5. SO_3^{2-} oxidation (% of initial amount 2 mM) by wheat or spinach chloroplasts (0.1 mg chlorophyll ml^{-1}) maintained for 1 hr in the absence or presence of superoxide dismutase (SOD) (100 units ml^{-1}) plus catalase (2500 units ml^{-1})

		Wheat		Spinach	
		Oxygen	Nitrogen	Oxygen	Nitrogen
− SOD − Catalase	Light	89.3 ± 9.0	44.7 ± 13.0	93.3 ± 16.8	19.2 ± 11.9
	Dark	57.2 ± 25.9	26.3 ± 15.3	22.8 ± 10.7	2.4 ± 2.3
+ SOD + Catalase	Light	33.6 ± 3.2	5.8 ± 1.1	9.1 ± 0.9	4.9 ± 1.0
	Dark	11.1 ± 1.8	0	2.9 ± 2.5	0

Data represent the mean of four experiments ± s.d.

(Table 5) or in the amount of sulphate recovered at the end of experiments (Fig. 1). At first, chloroplasts were stirred in the presence of 2 mM Na_2SO_3 without the addition of superoxide dismutase and catalase. A slight autooxidation of Na_2SO_3 always occurred in the solution prior to use. The amount of sulphate by autooxidation corresponded with the amount found in experiments carried out with boiled chloroplasts maintained under N_2 in the dark. This value was systematically substracted from all other results. It appeared that the presence of oxygen was not sufficient to lead to rapid sulphite oxidation; oxidation was low (*ca* 2% of initial sulphite) in the case of boiled chloroplasts maintained under O_2 in the dark. Nevertheless, the oxidation rate increased greatly when boiled chloroplasts were illuminated; 30–60% of initial sulphite was oxidized under these conditions. This indicated that the non-enzymatic oxidation of sulphite was stimulated by chloroplast pigments as reported elsewhere [9, 14]. On the other hand, sulphate formation was studied in fresh chloroplasts (Table 5). In the case of chloroplasts maintained under O_2 in the light, sulphite was almost completely transformed. Under other experimental conditions, sulphite oxidation was slower. Under N_2, photosynthetic O_2 evolved in the light would be involved in sulphite oxidation and there was probably sufficient residual oxygen in the chloroplast suspension and reaction medium to account for the oxidation observed in the dark. It is obvious that a part of this light-dependent sulphite oxidation was induced through the electron transport system in chloroplasts. To prevent this process, superoxide dismutase and catalase were added to scavenge O_2 radicals formed by chloroplasts and to destroy H_2O_2 produced by contaminating envelope-free chloroplasts [7]. The results of experiments carried out in this manner are shown in Table 5. Sulphite oxidation was largely decreased by the addition of superoxide dismutase (100 units ml^{-1}) and catalase (2500 units ml^{-1}). There was no oxidation under N_2 in the dark, but significant under all other conditions. Wheat chloroplasts possessed a higher capacity to oxidize sulphite than spinach; sulphite oxidation in the dark was *ca* one-third of that under light. Figure 1 illustrates the time-course of sulphite oxidation by wheat and spinach chloroplasts in the presence of superoxide dismutase and catalase, and corroborates previous observations. In all cases, sulphite oxidase activities appeared to be significant. Under O_2 in the light, maximum sulphite oxidation, calculated from Fig. 1 was *ca* 8.8 μmol hr^{-1} mg Chl^{-1} for wheat chloroplasts, and 2.7 μmol hr^{-1} mg Chl^{-1} for spinach.

In conclusion, it was previously established that intact wheat chloroplasts were able to oxidize S^0 into sulphate via the biosynthesis of thiosulphate and sulphite [4, 5].

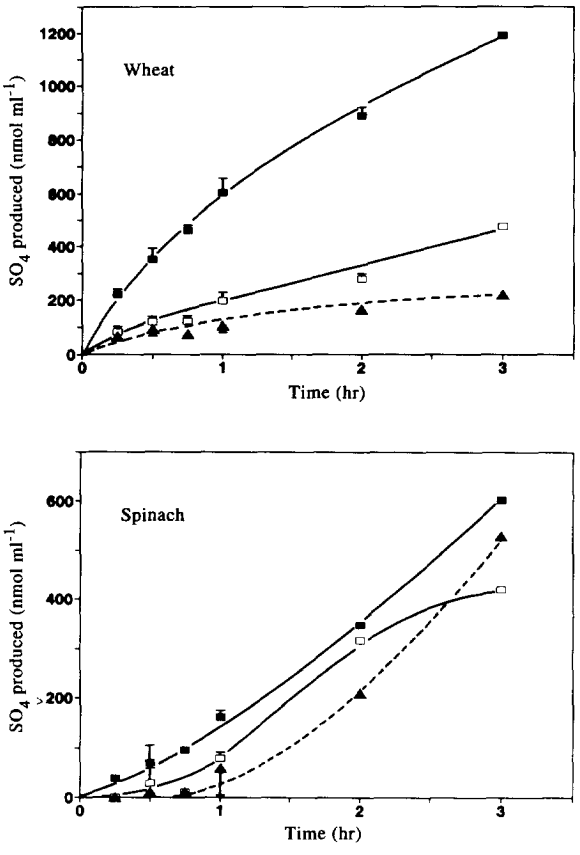

Fig. 1. Time-course of sulphite oxidation (initial concentration, 2 mM) by fresh chloroplasts from wheat (a) or spinach (b) leaves (0.1 mg chlorophyll ml^{-1}) in the light under O_2 (■) or under N_2 (▲) or in the dark under O_2 (□). Superoxide dismutase (100 units ml^{-1}) and catalase (2500 units ml^{-1}) were added to the reaction mixture. Experiments were done in triplicate.

The present results show that the same chloroplast process exists in spinach, since we have characterized five S compounds synthesized and labelled from $^{35}S^0$. The pathway of S^0 metabolism has not been elucidated unambiguously, since cysteine synthesis could be explained by sulphite or sulphate reduction. However, sulphite oxidation was observed in spinach chloroplasts as well as in those of wheat. This was achieved in part by slight autooxidation of Na_2SO_3, in part by a non-enzymatic process stimulated by chloroplast pigments, in part by the involvement of the photosynthetic electron transport chain, and finally by an intrinsic chloroplast system having sulphite oxidase activity.

EXPERIMENTAL

Wheat chloroplasts were prepd as described previously [5]. Spinach leaves (*Spinacia oleracea*) were purchased from a local market. Leaves were homogenized with 5 vols of isolation medium (10 mM tricine–NaOH buffer, 0.3 M sucrose, pH 7.6). After filtration through butter muslin, the suspension was spun (10 min, 1500 *g*). The pellet was resuspended in isolation medium and layered on top of a discontinuous sucrose gradient, 2, 1.75, 1.5 and 1 M [15]. After centrifugation (50 min, 50000 *g*), the intact chloroplast layer was recovered between 1.75 and 1.5 M, diluted with isolation medium containing 2 mM $MgCl_2$ and centrifuged (10 min, 6000 *g*). The pellet containing the chloroplasts was suspended in the reaction medium (0.33 M sorbitol, 50 mM Hepes, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, pH 7.6). For S^0 metabolism expts, spinach or wheat chloroplasts were shaken in the presence of 5 mM $NaHCO_3$ and $^{35}S^0$ (Amersham, France) as a suspension in Tween 80. Chlorophyll concn, estimated according to ref. [16], was *ca* 0.1 mg ml^{-1}. Chloroplast integrity was checked as described in ref. [5]. The initial concn of $^{35}S^0$ was 2 μ atom ml^{-1} (30.6 kBq mg^{-1} S) in expts carried out to determine the rate of S^0 metabolism. In the case of HPLC analysis of derivative S compounds, 5–11 μ atom ml^{-1} $^{35}S^0$ (4.8–11.1 MBq mg^{-1} S) was added to chloroplast suspensions. Either fr. isolated or boiled plastids were treated with S^0 at 18° for 2 hr. Samples were incubated in O_2 or N_2 either in the light (800 μmol m^{-2} s^{-1}) or in the dark. Expts were stopped by rapid centrifugation to pellet chloroplast lamellae and the supernatants were used for subsequent analyses. Radioactivity was determined by liquid scintillation counting and SO_4^{2-} radioactivity was counted after pptn with $BaCl_2$. Before turbidimetric SO_4^{2-} determination according to ref. [17], protein–pigment complexes solubilized with Tween 80 were pptd with 0.5 M TCA [3]. The determination of other S compounds was carried out by HPLC of their fluorescent monobromobimane derivatives according to ref. [18]. Sepn was achieved on an Ultrasphere ODS 5 μm analytical column as described previously [5]. Fluorescence detection was carried out with a fluorimeter (390 nm excitation, 480 nm emission) and radioactivity was measured using an in-line flow-through scintillation counter. The determination of S compounds was done with the help of standards which were analysed under the same chromatographic conditions individually and then in admixture. Standards were also added to samples to remove all ambiguity with regard to peak designations. Sulphate which does not react with monobromobimane was not detected by fluorimetry but could be detected as a radioactive peak at the beginning of the radiochromatogram. Each result was corrected for decay, and the labelling of S compounds was calculated at the time of assay. The efficiency of the radioactivity monitor was measured as described by Berthold using injection of known quantities of ^{35}S-labelled substances: $Na_2{}^{35}SO_4$ and L-[^{35}S] cysteine (Amersham, France) and estimated to be 0.60 for sulphate and 0.68 for S amino acids. Isotopic exchange was controlled by the addition of $^{35}S^0$ to standard solns of thiosulphate, sulphite or sulphate and analysis of labelling of the S compounds obtained. For sulphite metabolism expts, intact chloroplasts resuspended in the reaction medium at 0.1 mg chlorophyll ml^{-1} were stirred in glass tubes at 18° in the presence of 5 mM $NaHCO_3$ and 2 mM Na_2SO_3. N_2 was flushed through

the reaction medium and sulphite soln in order to avoid autooxidation. Expts were carried out under O_2 or N_2 in the light or dark or with fr. or boiled chloroplasts. Superoxide dismutase (100 U ml^{-1}) and catalase (2500 U ml^{-1}) were added in some expts. Catalase activity was defined according to Sigma and superoxide dismutase activity according to ref. [19]. Samples were removed from the reaction mixt. at different times over a 3 hr incubation time. Before turbidimetric determination of SO_4^{2-} concn, lamellar membranes were pelleted by centrifugation.

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