Maleimides stimulate oxygen reduction in illuminated thylakoids

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Abstract ***N*-ethylmaleimide (NEM) and *N*,*N*′-(1,4-phenylene)-dimaleimide (PDM) were discovered to stimulate light-induced oxygen uptake in isolated thylakoids, and PDM provided the same stimulation at one order less concentrations. Oxygen uptake rate increased promptly after NEM or PDM addition to thylakoids. The inhibitors of photosynthetic electron transport as well as catalase decreased this rate close to zero, whereas ascorbate increased it almost three-fold. Dithiothreitol suppressed oxygen uptake stimulated by NEM. NEM stimulated light-induced reduction of cytochrome *c*, and this stimulation was suppressed by superoxide dismutase. It was concluded that NEM and PDM being reduced can effectively reduce molecules O_2 producing superoxide radicals.**

Key words: Thylakoid; Maleimide; Oxygen uptake; Superoxide radical

1. Introduction

Sulfhydryl alkylating agent *N*-ethylmaleimide (NEM) is broadly used in biochemistry to investigate the mechanisms of enzyme activity [1–3], and to reveal the homology of the proteins possessing similar functions [4]. Sulfhydryl groups are considered as the target of NEM attack, and the inhibition of enzyme activity under treatment of NEM implies the presence of sulfhydryl groups in the active center. Maleimides also are widely used in chemical studies for investigation of mechanisms of polymerization [5–7] and photopolymerization [8]. They also found an outlet in medicine [9]. In previous years NEM was successfully used in the studies of energy transformation in chloroplasts. NEM was found to inhibit specifically some reactions in ATPase [10] as well as an activity of ferredoxin–nicotinamide adenine dinucleotide phosphate reductase [11]. In all the cases the thylakoids were treated with NEM in the dark. The possible interaction of NEM with components of thylakoids in the light was not considered. In the present work we found that NEM as well as its lipophilic analog *N*,*N*′-(1,4-phenylene)dimaleimide (PDM) may readily accept the electrons from photosynthetic electron transport chain (PETC) of thylakoids in the light and transfer these electrons to oxygen providing the generation of superoxide radicals.

2. Materials and methods

Thylakoids from leaves of pea plants, grown in a greenhouse at temperature 23/17°C (day/night) and under natural illumination, were isolated as described [12]. The final thylakoid pellet was suspended in 1–2 ml of medium containing 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 10 mM HEPES–KOH (pH 7.6) to chlorophyll (Chl) concentration approximately 1 mg ml^{-1}, and stored on ice. The obtained thylakoid preparations were essentially catalase-free that was checked with the measurements of H_2O_2 decomposition.

Oxygen concentration changes in a stirred thylakoid suspension (2.2 ml) were measured at 20°C in a thermostated vessel with a Clark-type oxygen electrode, using the reaction media containing 0.1 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM mesencephalon (Mes)–KOH/glycine (pH 5.0), or 25 mM Mes–KOH (pH 6.5), or 25 mM HEPES–KOH (pH 7.8). Light-induced quenching of 9-aminoacridine (9AA) fluorescence was measured in the above medium at pH 7.8. The fluorescence was excited by mercury lamp light passed through a 365 nm interference filter, and UFS-6, UFS-8, and gray NS-3 filters (Russia). It was recorded by a photomultiplier, which was protected by GS-12, and two SZS-22 filters (Russia) against exciting and actinic lights. Actinic illumination was provided by a 150 W halogen lamp the light of which was filtered through a KS-15 cutoff filter (Russia) and a heat absorbing filter. Photoreduction of ferricytochrome *c* (ferri-Cyt *c*) by thylakoids was measured at 20°C as an increase in absorption at 550 nm with a reference at 540 nm using a dual wavelength spectrophotometer (Hitachi 553, Japan). The difference absorption coefficient of ferro-/ferri-Cyt *c* at 550 nm was taken as 19 mM^{-1} cm^{-1}. The reaction mixture (3 ml) contained 0.1 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM HEPES–KOH (pH 7.8), 40 μM Cyt *c*, and 0.1 mM ethylenediamine tetraacetic acid (EDTA). The concentration of thylakoids in all the assays was 15 μg Chl ml^{-1}. Chl concentration was determined in 95% ethanol extracts [13].

The reaction medium in all experiments was illuminated with light, which passed through a red filter ($\lambda > 600$ nm); photon flux density measured using Li-Cor quantum meter (model LI-250) was 450 μE s^{-1} m^{-2}. The light was switched on in 2 min after NEM, PDM, or methyl viologen (paraquat) (MV) and other additions were inserted into a reaction mixture, and the illumination kept on until steady rate of oxygen uptake was established that usually took no more than 2 min.

3. Results

Fig. 1 shows that NEM as well as PDM stimulate light-induced oxygen uptake in the thylakoid suspension. The addition into the reaction mixture of either gramicidin D (Gr D) (Fig. 1) or nigericin (not shown) highly increased the oxygen uptake rate. This implies that the electron transport in the presence of maleimides is decelerated by a lumen acidification coupled to the electron transfer along the ETC. An increase in the extent of light-induced quenching of 9AA fluorescence in

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Abbreviations: Chl, chlorophyll; Cyt *c*, cytochrome *c*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, dinitrophenylether of 2-iodo-4-nitrothymol; DTT, 1,4-dithio-DL-threitol; Gr D, gramicidin D; MDHA, monodehydroascorbate radical; MV, methyl viologen (paraquat); NEM, *N*-ethylmaleimide; PDM, *N*,*N*′-(1,4-phenylene)dimaleimide; PETC, photosynthetic electron transport chain; SOD, superoxide dismutase; 9AA, 9-aminoacridine

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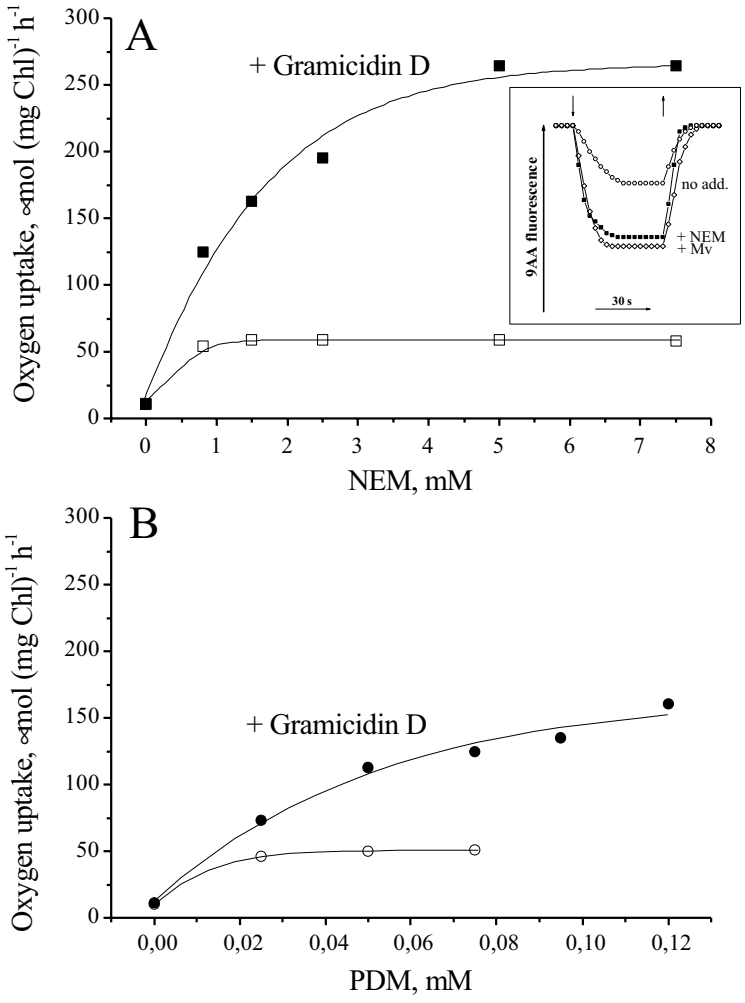

Fig. 1. The dependencies of the rates of light-induced oxygen uptake in isolated thylakoids on the concentration of NEM (A) and PDM (B). 1 μM Gr D was present in reaction medium where it is indicated; pH 7.8. The insert in A shows the effects of 5 mM NEM as well as of 0.1 mM MV, added into thylakoid suspension (pH 7.8), on the light-induced quenching of 9AA fluorescence. Length of the thick upward arrow represents 100% of 9AA fluorescence yield in the dark in the presence of NEM or MV as well as in their absence; the thin downward and upward arrows show the switching on and off of the actinic light respectively.

the presence of NEM (Fig. 1, insert) indicated a gain in formation of proton gradient across the thylakoid membrane in this case. This gain was obviously the result of lifting restrictions, which limit the electron transport in washed thylakoids in the absence of any acceptors besides oxygen usually at the stage of electron transfer from acceptor side of photosystem I to O_2. Just due to lifting such restrictions the photosynthetic electron transport is accelerated by MV, which also increases light-induced quenching of 9AA fluorescence (Fig. 1, insert).

In the presence of the uncouplers the electron transport rate was saturated at 5 mM NEM, while PDM stimulated oxygen uptake at much lower concentrations (Fig. 1). The stimulation of oxygen uptake by NEM and PDM was observed in pH range 5.0–8.0 (not shown), and the rate of oxygen uptake promptly increased when either NEM or PDM were added during illumination of the thylakoids (Fig. 2). The same was observed also in response to addition of MV, low-potential acceptor at photosystem I promptly transferring electrons to oxygen. The presence of 1,4-dithio-DL-threitol (DTT) in the reaction mixture increased this effect of MV while strongly abolishing the stimulation of the rate of oxygen uptake by NEM or PDM (Fig. 2). It is seen from Table 1 that DTT really increased the oxygen uptake rate with MV, while it decreased this rate in the presence of NEM. DTT is usually added to defend the sulfhydryl groups of proteins from oxidation, and in particular, from interaction with NEM. Reaction of DTT with the maleimide group generates a stable 3-thiosuccinimidyl ether linkage, and maleimides lose capacity to react with sulfhydryl groups of proteins. It was an important finding that the inhibitory effect of DTT on the light-induced oxygen uptake with NEM very weakly depended on the order of additions of NEM and DTT into the reaction mixture (Fig. 3).

To scrutinize additionally the expected interaction of NEM with components of thylakoid membrane as possible reason of its effect on light-induced oxygen uptake we compared the oxygen uptake in thylakoids incubated either with or without NEM 10 min in the dark or 5 min in the light, and washed after the incubation. We found that in all the cases the oxygen uptake rates were low and very close (not shown).

The data presented in Table 1 show that the inhibitors of

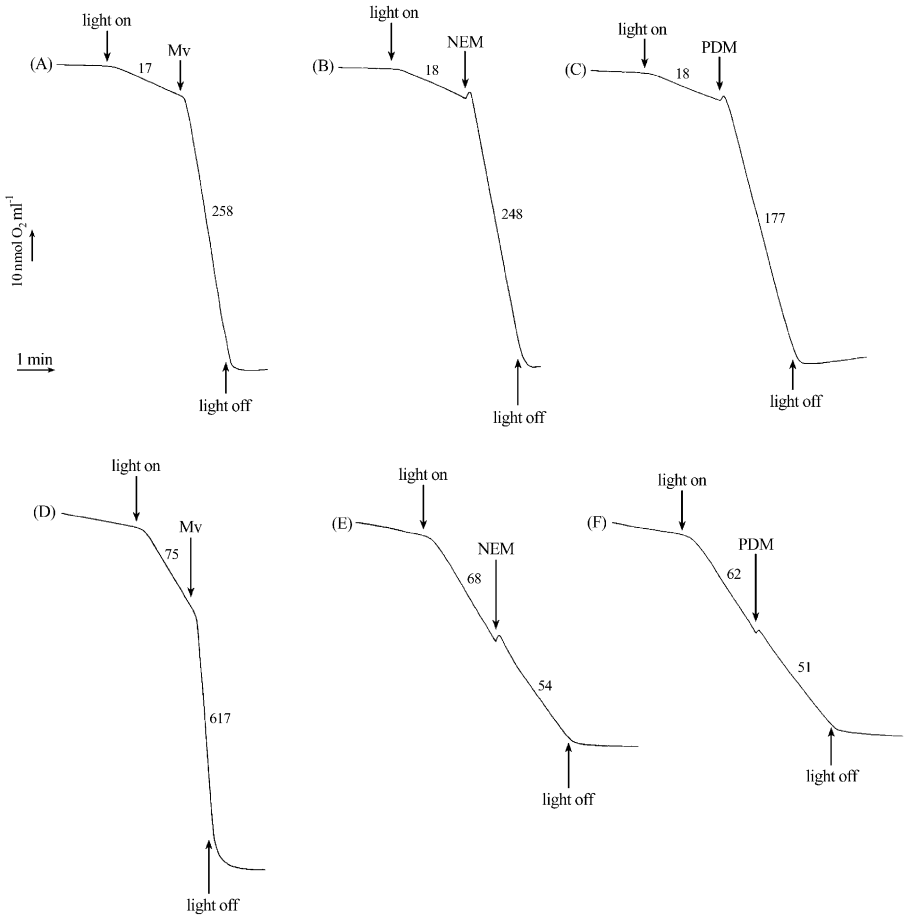

Fig. 2. An influence of additions of 0.1 mM MV (A, D), 5 mM NEM (B, E), 120 μM PDM (C, F) into suspension of isolated thylakoids under illumination on oxygen uptake rate. In D, E, and F 5 mM DTT was present in the reaction medium before illumination. Numbers near the curves are the rates of oxygen uptake in μmol (mg Chl)$^{-1}$ h^{-1}. The reaction mixture, pH 7.8, also contained 1 μM Gr D.

photosynthetic electron transport, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and dinitrophenylether of 2-iodo-4-nitrothymol (DNP-INT), as well as catalase decrease the rate of oxygen uptake in the presence of NEM almost to zero, while ascorbate increases it. The inhibitory effect of DNP-INT stated additionally that the electrons reaching NEM were transferred between photosystems with plastoquinone participation.

These results could be, if oxygen uptake resulted from the reactions, occurring in the presence of low-potential acceptors at photosystem I [14]:

$$H_2O \quad 2e^- + {}^1/_2O_2$$
(water oxidation in PSII, reaction 1)

$$2\ \text{acceptor}^{ox} + 2e^- = 2\ \text{acceptor}^{red}$$
(acceptor reduction, reaction 2)

$$2O_2 + 2\ \text{acceptor}^{red} = 2O_2^{\bullet -} + 2\ \text{acceptor}^{ox}$$
(oxygen reduction, reaction 3)

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ = H_2O_2 + O_2$$
(dismutation, reaction 4)

Table 1
The comparative characteristics of light-induced oxygen uptake in isolated thylakoids in the presence of NEM and MV

Additions into thylakoid suspension	Oxygen uptake rate[a], μmol (mg Chl)$^{-1}$ h^{-1} in the presence of	
	NEM, 5 mM	MV, 0.1 mM
–[b]	260 ± 6	295 ± 5
DCMU, 10 μM	2 ± 1	3 ± 2
DNP-INT, 5 μM	10 ± 3	12 ± 2
Catalase, 500 U ml^{-1}	3 ± 1	2 ± 1
DTT, 5 mM	53 ± 4	611 ± 7
Ascorbate, 5 mM	669 ± 9	710 ± 8
Ascorbate, 5 mM+catalase, 500 U ml^{-1}	308 ± 12	332 ± 10

The reaction mixture, pH 7.8, also contained 1 μM Gr D.
[a]The data from typical experiment ± a mean absolute error from three–four measurements.
[b]The rates in the first row in different thylakoid preparations ranged from 210 to 305 μmol (mg Chl)$^{-1}$ h^{-1} for NEM, and from 240 to 350 μmol (mg Chl)$^{-1}$ h^{-1} for MV.

In this case, the stoichiometry between electron transport and oxygen uptake is such that one molecule of O_2 consumed corresponds to transfer $4e^-$ from water to acceptor. When catalase is present in the reaction mixture the resulted change of oxygen concentration there has to be zero:

$H_2O_2 \quad H_2O + {}^1/_2O_2$ (catalase action, reaction 5).

The effect of catalase in Table 1 is in total correspondence with this. Thus, all the data implied that electrons, which triggered oxygen uptake in the presence of NEM, were derived from water in photosystem II, and that hydrogen peroxide was the final product.

The steep increase in the oxygen uptake rate in the presence of ascorbate pointed to superoxide radical being formed during the light-induced process. This evidenced that the oxygen uptake was the result of one-electron dioxygen reduction. As the trap of superoxide radicals, ascorbate prevents their dismutation [15], and reaction 4 is replaced with reaction 6:

$2O_2^{\bullet -} + 2\mathrm{AscH} + 2H^+ = 2\mathrm{MDHA} + 2H_2O_2$ (reaction 6).

This has to lead to an increase of oxygen uptake rate in the presence of ascorbate, theoretically three-fold according to the above equations. In reality (Table 1), ascorbate increased oxygen uptake rate with NEM by 2.57 times, while with MV by 2.4. The divergence from theoretical value might result from both, concurrent dismutation (reaction 4) and/or simultaneous MDHA (monodehydroascorbate radical) reduction accompanied by oxygen evolution [16]. The addition of catalase in the presence of ascorbate substantially decreased the rate, as this has to be according to stoichiometry of the reactions. The stimulatory effect of DTT on oxygen uptake in the presence of MV (Fig. 2, Table 1) was evidently explained by that DTT being reductant could react with superoxide radical in the same manner as ascorbate, and that DTT did not react specifically with MV in contrast to such a reaction with NEM.

The NEM-dependent generation of superoxide radicals under illumination of isolated thylakoids was proved also by stimulation of Cyt *c* reduction by NEM, and by inhibition of this stimulation by superoxide dismutase (SOD) (Table 2). It may be seen from Table 2 that NEM stimulated Cyt *c* reduction in a greater degree than MV.

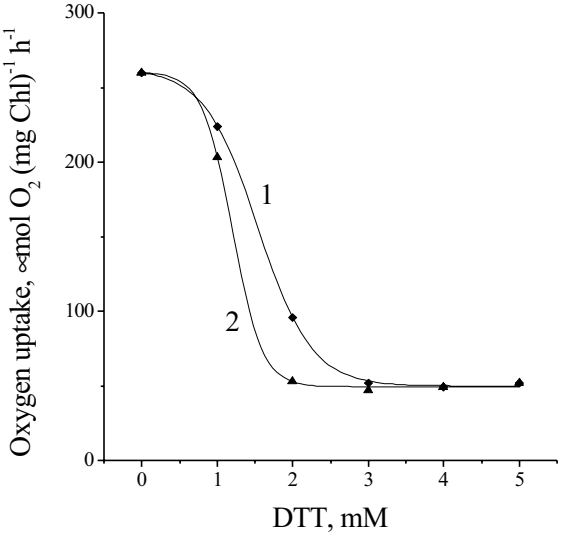

Fig. 3. An influence of DTT addition on the rate of light-induced oxygen uptake in isolated thylakoids in the presence of NEM. DTT was added before (1) or after NEM (2). The reaction mixture, pH 7.8, also contained 1 μM Gr D.

Table 2
Effects of NEM and MV on Cyt *c* reduction in the isolated thylakoids

Additions into suspension	Cyt *c* reduction rate[a], μmol (mg Chl)$^{-1}$ h^{-1}
–[b]	15 ± 2
NEM, 5 mM	237 ± 5
NEM, 5 mM+SOD, 300 U ml^{-1}	21 ± 3
MV, 0.1 mM	177 ± 3

The reaction mixture, pH 7.8, also contained 1 μM Gr D.
[a]The data from typical experiment ± a mean absolute error from three–four measurements.
[b]The rate in the first row in different thylakoid preparations ranged from 8 to 20 μmol (mg Chl)$^{-1}$ h^{-1}.

4. Discussion

It is elicited from obtained data that in the light NEM and PDM are capable to accept electrons from reduced component(s) of PETC ranging 'after' plastoquinone, most probably at acceptor side of photosystem I, and transfer them to dioxygen providing the production of superoxide radicals.

The characteristics of light-induced oxygen uptake with NEM were very close to those with MV, whose herbicide action results mostly from reactive oxygen species (ROS) production in the chloroplasts in the light. Some quantitative differences in the effects of NEM and MV might be explained by the higher capability of reduced MV molecules preserving one positive charge to reduce the just formed negatively charged superoxide radicals. Such a reduction does not change the stoichiometry between electron transport and oxygen uptake, however does decrease the lifetime of superoxide radical. The longer lifetime of superoxide in the presence of NEM could be responsible for the higher stimulation of oxygen uptake by ascorbate (Table 1) as well as for the higher rate of superoxide-dependent Cyt *c* reduction in the presence of NEM than in the presence of MV (Table 2).

The inhibitory effect of DTT on the light-induced oxygen uptake stimulated by NEM is evidently a result of an interaction of DTT with NEM. The weak dependence of DTT effect on the order of their additions into reaction mixture (Fig. 3) revealed that possible modification of sulfhydryl groups of proteins might hardly be reason of the observed oxygen uptake stimulation by NEM. If the inhibitory effect of DTT was connected with prevention of NEM interaction with sulfhydryl groups of proteins, then DTT addition after NEM might be either without such an effect or with appreciably diminished effect. The opposite result, namely the necessity of a slightly higher DTT concentration to reach a maximal inhibition when it was added before NEM (Fig. 3), is possible a result of oxidation of some DTT molecules by thylakoid membrane components in the dark, and thus of a decrease of its operative concentration. The prompt stimulation of the rate of oxygen uptake in the response to NEM or PDM addition into thylakoid suspension (Fig. 2) as well as the results of experiments with incubated thylakoids also contradicted the presumption that NEM interaction with thyla-

koid membrane components brought about the effects described in this work.

Thus, the results of our work show that once maleimides are reduced they acquire the ability to produce ROS, superoxide radical and hydrogen peroxide. The use of such maleimide as NEM as herbicide seems non-actual as its concentrations for the same effects are much higher than concentrations of MV. However, the modification of molecule structure may highly increase the effectiveness of maleimide derivatives, the same increase in the rate of oxygen uptake was observed at PDM concentrations one order less than NEM concentrations (Fig. 1). Possibly, this resulted from higher lipophility of PDM. This would allow it to accept more effectively electrons from the PETC components buried in the thylakoid membrane. Some advantage may also come from the fact that the lifetime of superoxide produced by maleimides may be longer than lifetime of ones produced by MV.

Maleimides already are widely used in the biochemical, chemical and medical studies (see Section 1), and the results of the present work may be important to stimulate additional consideration of their action from the point of view of probable production of ROS in specific cases, in biological objects having redox chains, or NAD(P)H-oxidases, or cytochrome *P*450, and in chemical reactions, where their reduction is possible.

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References

[1] Bize, I., Guvenc, B., Buchbinder, G. and Brugnara, C. (2000) J. Membr. Biol. 177, 159–168.
[2] Marre, M.T. and Albergoni, F. (1998) Plant Physiol. 116, 681–686.
[3] Duhe, R.J., Nielson, M.D., Dittmann, A.H., Villacres, E.C., Choi, E-J. and Storm, D.R. (1994) J. Biol. Chem. 269, 7290–7296.
[4] Wenander, R., Anderson, C. and Morgenstern, R. (1994) J. Biol. Chem. 269, 71–96.
[5] Flel, D. and Vukovic, R. (1995) J. Macromol. Sci. Pure Appl. Chem. 32, 1461–1469.
[6] Kohli, P., Scranton, A.B. and Blanchard, G.J. (1998) Macromolecules 31, 5681–5689.
[7] Curran, D.P., Geib, S. and DeMello, N. (1999) Tetrahedron 55, 5681–5687.
[8] Obata, A.T., Shimo, T., Yasutake, M., Shinmyoz, T., Kawaminami, M., Yoshida, R. and Somekawa, K. (2001) Tetrahedron 57, 1531–1541.
[9] Kalgutkar, A.S., Crews, B.C. and Marnett, L.J. (1997) Adv. Exp. Med. Biol. 407, 79–85.
[10] McCatry, R.E. (1977) in: Encyclopedia of Plant Physiology, New series (Trebst, A. and Avron, M., Eds.), Vol. 5, pp. 437–447, Springer, Heidelberg.
[11] Shahak, Y., Crowther, D. and Hind, G. (1981) Biochim. Biophys. Acta 636, 234–243.
[12] Khorobrykh, S.A. and Ivanov, B.N. (2002) Photosynth. Res. 71, 209–219.
[13] Lichtenthaler, H.K. (1987) Methods Enzymol. 148, 350–382.
[14] Allen, J.F. (1977) in: Superoxide and Superoxide Dismutases (Michelson, A.M., McCord, J.M. and Fridovich, I., Eds.), pp. 417–436, Academic Press, London.
[15] Allen, J.F. and Hall, D.O. (1973) Biochem. Biophys. Res. Commun. 52, 856–862.
[16] Ivanov, B.N. (2000) Free Radic. Res. 33, 217–227.