ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 4, pp. 384-394. © Pleiades Publishing, Inc., 2006.
Original Russian Text © V. D. Samuilov, D. B. Kiselevsky, S. V. Sinitsyn, A. A. Shestak, E. M. Lagunova, A. V. Nesov, 2006, published in Biokhimiya, 2006, Vol. 71, No. 4, pp. 481-492.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM05-223, February 12, 2006.

H_2O_2 Intensifies CN^--Induced Apoptosis in Pea Leaves

V. D. Samuilov*, D. B. Kiselevsky, S. V. Sinitsyn, A. A. Shestak, E. M. Lagunova, and A. V. Nesov

Department of Physiology of Microorganisms, Biological Faculty, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3807; E-mail: vdsamuilov@mail.ru

Received October 11, 2005
Revision received November 16, 2005

Abstract—H_2O_2 intensifies CN^--induced apoptosis in stoma guard cells and to lesser degree in basic epidermal cells in peels of the lower epidermis isolated from pea leaves. The maximum effect of H_2O_2 on guard cells was observed at 10^{-4} M. By switching on non-cyclic electron transfer in chloroplasts menadione and methyl viologen intensified H_2O_2 generation in the light, but prevented the CN^--induced apoptosis in guard cells. The light stimulation of CN^- effect on guard cell apoptosis cannot be caused by disturbance of the ribulose-1,5-bisphosphate carboxylase function and associated $OH^\cdot$ generation in chloroplasts with participation of free transition metals in the Fenton or Haber–Weiss type reactions as well as with participation of the FeS clusters of the electron acceptor side of Photosystem I. Menadione and methyl viologen did not suppress the CN^--induced apoptosis in epidermal cells that, unlike guard cells, contain mitochondria only, but not chloroplasts. Quinacrine and diphenylene iodonium, inhibitors of NAD(P)H oxidase of cell plasma membrane, had no effect on the respiration and photosynthetic O_2 evolution by leaf slices, but prevented the CN^--induced guard cell death. The data suggest that NAD(P)H oxidase of guard cell plasma membrane is a source of reactive oxygen species (ROS) needed for execution of CN^--induced programmed cell death. Chloroplasts and mitochondria were inefficient as ROS sources in the programmed death of guard cells. When ROS generation is insufficient, exogenous H_2O_2 exhibits a stimulating effect on programmed cell death. H_2O_2 decreased the inhibitory effects of DCMU and DNP-INT on the CN^--induced apoptosis of guard cells. Quinacrine, DCMU, and DNP-INT had no effect on CN^--induced death of epidermal cells.

DOI: 10.1134/S0006297906040067

Key words: apoptosis, reactive oxygen species, chloroplasts, mitochondria, plasma membrane, *Pisum sativum* L.

Programmed cell death (PCD) is a physiological response to both internal and external signals; the cell dies, but inflicts no damage on its environment. Both chemical and physical affects compared to natural stimuli have broader possibilities, because they cause massive synchronous death of cells, thereby facilitating the following analysis of results. We used cyanide [1], a PCD inducer in plants, which causes internucleosomal cleavage of nuclear DNA [2, 3]. CN^- possesses multiple effects: it inhibits mitochondrial cytochrome oxidase, catalase, peroxidases including ascorbate peroxidase of chloroplasts, and Cu,Zn-superoxide dismutase [4], and inactivates ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) [5].

As shown by optical microscopy, CN^- causes nuclear fragmentation and destruction in cells of epidermis isolated from pea leaves [1]. Epidermis is a monolayer composed of the stoma guard cells (GC) and the basic epidermal cells (EC), which differ in structure and functions: GC contain both chloroplasts and mitochondria, whereas EC contain mitochondria only. Light significantly accelerated the CN^--induced destruction of GC nuclei and had no effect on destruction of EC nuclei [1]. Electron microscopy of GC ultrastructure has demonstrated its expressed CN^--induced dynamics [6]. Both chromatin condensation and margination became visible in GC already after 1 h of incubation of isolated epider-

Abbreviations: BQ) *p*-benzoquinone; DAPI) 4′,6-diamidino-2-phenylindole dihydrochloride; DCF) 2′,7′-dichlorofluorescein; DCFH) 2′,7′-dichlorofluorescin (diacetate); DCMU) 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DNP-INT) iodonitrothymol dinitrophenyl ester; DPI) diphenyleneiodonium; EC) basic epidermal cells; FeCy) potassium ferricyanide; GC) stoma guard cells; LS) leaf slices; NBT) nitroblue tetrazolium; PCD) programmed cell death; ROS) reactive oxygen species; Rubisco) ribuloso-1,5-bisphosphate carboxylase/oxygenase; TMPD) N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

* To whom correspondence should be addressed.

mis with CN^-. After 6 h of incubation with CN^- the main volume of GC was filled with vacuoles, the cytoplasm concentrated in a thin parietal layer, the nucleus became multibladed, was outstretched in thin plasmatic bands, but, despite the loss of nuclear envelope integrity, maintained a separate structure. Mitochondria and chloroplasts were in direct contact with chromatin on exposed, membraneless nuclear areas. Mitochondria swelled. Chloroplasts, alike the cell nucleus, lost membrane continuity, but did not swell and maintained stroma and integrity of the thylakoid system. The pattern of ultrastructural changes is indicative of apoptotic character of CN^--induced GC death [6].

CN^--induced destruction of GC and EC nuclei was prevented by antioxidants, such as α-tocopherol, butylated hydroxytoluene, and mannitol, as well as under anaerobiosis [7, 8]. The electron acceptors, such as *p*-benzoquinone, menadione, and methyl viologen, which maintain the Hill reaction in chloroplasts, inhibited CN^--induced apoptosis of GC. Light activation of GC apoptosis was withdrawn by 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) (an inhibitor of electron transport in Photosystem II), stigmatellin, and iodonitrothymol dinitrophenyl ester (DNP-INT) (inhibitors of plastoquinol oxidation by the Rieske FeS-protein in the *o* site of cytochrome b_6f complex of chloroplasts). The process was prevented by the protein kinase inhibitor staurosporin. It has been concluded that initiation of GC apoptosis depends on combined effect of two factors: reactive oxygen species (ROS) and functionally active plastoquinone in the *o* site of cytochrome b_6f complex [7, 8]. Experiments with pea mutants have demonstrated that both light stimulation of CN^--induced GC apoptosis and its withdrawal by DCMU are associated with functioning of Photosystem II [9].

Light dependence of PCD has been demonstrated for the *Arabidopsis thaliana* mutants lsd1 [10] and acd11 [11], as well as for the maize mutant lls1 [12]. They are so-called lesion mimic mutants characterized by spontaneous PCD associated with defects in genes regulating the PCD upon hypersensitive response, a defensive reaction of plants to infective agents, which can develop in the absence of pathogens [13]. Illumination is also required for the PCD of *A. thaliana* induced by either the mycotoxin fumonisin B1 [14] or short-wave UV [15]. A decrease in the level of the chloroplast protein DS9, a homolog of metalloproteinase FtsH, correlates with accelerated cell death in tobacco leaves infected by tobacco mosaic virus [16]. The above-cited data are evidence for involvement of chloroplasts in apoptosis.

Mitochondria in mammals including humans are suppliers of a series of pro-apoptotic components, such as cytochrome *c*, flavoprotein AIF (apoptosis-inducing factor), and endonuclease G [17]. In plant PCD cytochrome *c* releases from mitochondria to cytoplasm (see review [18]). However, it is unclear whether cytochrome *c* is involved in plant PCD: it cannot be ruled out that withdrawal of cytochrome *c* is just a consequence of mitochondrial destruction [19]. A Mg^{2+}-dependent nuclease was found in intermembrane space of plant mitochondria; possibly, this nuclease is functionally related to animal endonuclease G [20]. There is some evidence for correlation between disturbance of mitochondrial membrane permeability with formation of giant pores (permeability transition) and PCD induction in plants, but these data require further molecular genetic studies [19].

Both in animals and plants PCD can be activated by ROS. The nature of cell responses is dependent on ROS concentration. High doses of ROS induce PCD, for example, via hypersensitive response, whereas low doses of ROS call forth induction of antioxidant enzymes (superoxide dismutase, catalase, and peroxidase) and cell cycle arrest [21]. Hydrogen peroxide plays the role of signal molecule in PCD, enhances cascade of mitogen-activated kinases (the mechanism of transcription factor activation and protective gene expression), induces expression of plasma membrane NADPH-oxidase, and induces stoma closing in plants [22, 23]. In the cell, H_2O_2 is preferably generated from one-electron reduction of O_2 with formation of $O_2^{\cdot-}$ superoxide anion-radical and its subsequent disproportionation by superoxide dismutase. The sources of $O_2^{\cdot-}$ in plant cells are the plasma membrane-associated NADPH-oxidase [22] and electron transport chains of chloroplasts [4] and mitochondria [24, 25]. Besides, H_2O_2 in plants is formed with involvement of cell wall-associated peroxidase and oxalate oxidase [22].

The aim of present work was to study the effect of exogenous H_2O_2 on CN^--induced PCD in pea leaf epidermis and to elucidate the nature of the ROS source, which is necessary for realization of this process. The data obtained shows that the more probable source of ROS in GC apoptosis is the cellular plasma membrane-associated NAD(P)H-oxidase, but not the electron transport chains of chloroplasts or mitochondria.

MATERIALS AND METHODS

The experiments were carried out on peels of lower leaf epidermis of pea (*Pisum sativum* L. cv Alpha) seedlings grown for 7-15 days under continuous illumination at 20-24°C [1]. Epidermis was separated with forceps and placed into distilled water. The infiltration method via epidermis incubation in vacuum for 1-2 min was used for rapid influx of added reagents into the cells. The samples were placed into polystyrene plates and incubated in distilled water with additives (the composition is given in legends for figures) at room temperature either in dark or under illumination with a luminescent lamp at the light intensity of ~1000 lx.

After the incubation, the samples were treated for 5 min with Battaglia fixative (mixture of chloroform, 96%

ethanol, glacial acetic acid, and 40% formaldehyde, 5 : 5 : 1 : 1). Thereupon the samples were washed in ethanol for 10 min for removal of the fixing mixture, incubated for 5 min in water, and stained with Carazzi's hematoxylin for 20 min. The stained peels were washed with tap water and examined in light microscope. The number of cells with destructed nuclei and lack of nuclei was determined from 300-500 examined cells (for each epidermal peel) [1].

The epidermis was fixed as described above and stained for 15 min with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (0.2 μM water solution) for fluorescence microscopy. The observations were done using the fluorescence microscope Carl Zeiss Axiovert 200M (Germany) at 360-380 nm in phase contrast mode.

Evolution and consumption of O_2 by pea leaf slices (LS) was measured using a closed Clark Pt-electrode. The incubation medium for LS (10 mg/ml) contained 10 mM HEPES-NaOH, pH 7.0, and 25 mM KCl. Chlorophyll concentration in LS corresponded to 45.3 μg/ml. White light of saturating intensity (~0.1 W/cm^2) was used in experiments. The content of chlorophylls *a* and *b* in LS was determined by extraction with 80% acetone (water solution) [26].

Fluorescence of 2′,7′-dichlorofluorescein (DCF) was measured using a VersaFluor fluorimeter (Bio-Rad, USA). Epidermis was fixed on its intact surface on a polystyrene plate, submerged into the solution of 50 μM 2′,7′-dichlorofluorescin (diacetate) (DCFH), incubated for 10 min in darkness, washed with distilled water, and placed into the sample cell with 25 mM HEPES-NaOH solution, pH 7.2. The excitation wavelength for DCF fluorescence was 485-495 nm, and the emission was registered at 515-525 nm.

All experiments were replicated 3-5 times. Most typical experimental data are given.

RESULTS

Effect of H_2O_2 on the CN^--induced destruction of GC and EC nuclei. CN^--induced destruction of nuclei in GC and EC was demonstrated earlier [1]. The loss of EC nuclei reached almost 100% already after 1-2 h of incubation of pea leaf epidermis peels. The destruction of GC nuclei developed slower and reached maximum after 20-24 h. Light activated the CN^--induced destruction of nuclei in GC, but not in EC [1]. Figure 1 demonstrates that the CN^--induced destruction of GC nuclei is enhanced by addition of H_2O_2 in darkness, as well as in light. Significant stimulation of the process was observed already at 10 μM concentration of H_2O_2, and almost 100% destruction of nuclei was achieved at 100 μM H_2O_2. Hydrogen peroxide did not induce nucleus destruction in the absence of CN^- even at 10-50 mM concentrations (Fig. 1).

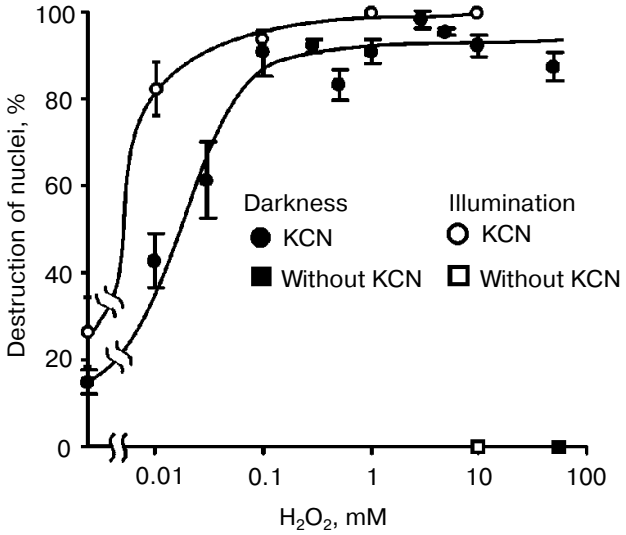

Fig. 1. Effect of H_2O_2 on CN^--induced destruction of GC nuclei in epidermis from pea leaves in the dark and in the light. Epidermis after infiltration with H_2O_2 was incubated for 30 min in the dark and then 2.5 mM KCN was added followed by incubation for 17 h in the dark or 14 h in the light with subsequent fixation and staining with hematoxylin.

The stimulatory effect of H_2O_2 on destruction of EC nuclei treated with 2.5 mM KCN was lower than on GC (data not shown); it was apparent at higher concentrations of H_2O_2 (100 μM and higher) and was absent in some samples of epidermis. Hydrogen peroxide (10 mM) in the absence of CN^- did not induce destruction of EC nuclei.

Figure 2 illustrates the data of optical (a) and fluorescence (DAPI staining) (b, c, d) microscopy observations of cells in epidermal peels. DAPI is a fluorescence dye penetrating the cells and binding to thymine/adenine-enriched sites in minor grooves of double-stranded DNA [27]. The morphology of EC nuclei was significantly changed after 30 min incubation with CN^- and H_2O_2 (Figs. 2b, II, and 2c, II): their bulging and deformation were observed, as well as separation into loculi induced by DNA fragmentation. The nuclei of EC disappeared after 2.5 h incubation with CN^- and H_2O_2 (Fig. 2, a and b, III). The structural alterations of GC nuclei developed more slowly. A multilocular structure formation in GC nuclei and their fragmentation were observed after 8 h incubation with CN^- and H_2O_2 (Fig. 2d, II).

Effect of Fe^{2+} + ascorbate on CN^--induced destruction of GC nuclei. In the presence of transition metals, such as iron and copper, ascorbate is a potent pro-oxidant [28, 29]. Dehydroascorbate, the product of ascorbate oxidation, is toxic and induces the oxidative stress [30, 31]. Figure 3a demonstrates that $FeSO_4$, ascorbate, and their combination *per se* do not induce destruction of GC nuclei. On the background of CN^- the combination of $FeSO_4$ and ascorbate slightly enhanced the destruction of nuclei. This effect developed both in the darkness and in

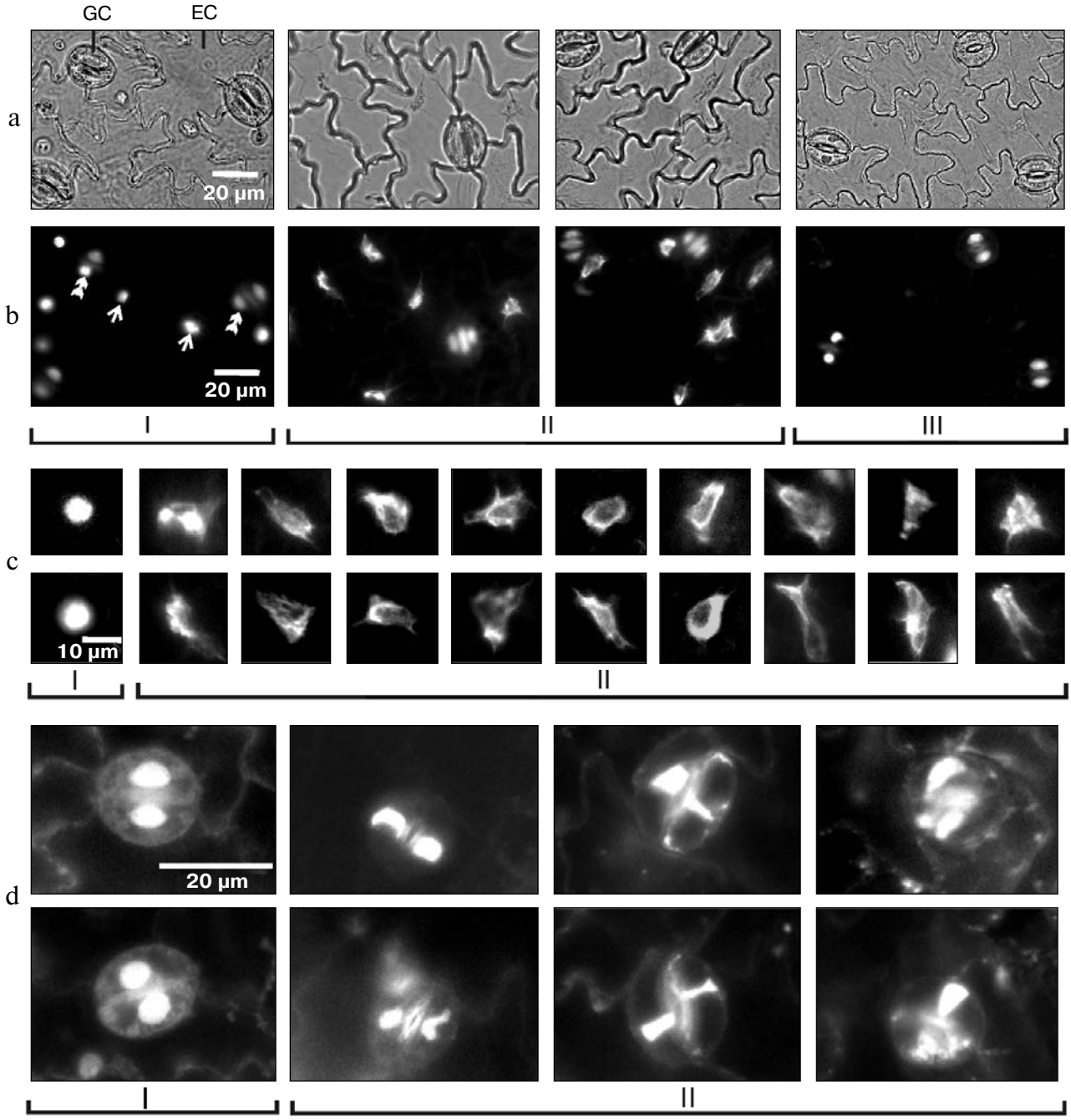

Fig. 2. Data of optical (a) and fluorescence microscopy (b, c, d) (DAPI staining) of EC, GC (a, b, d), and nuclei of EC (c) in pea leaf epidermis: I) control without additives; II, III) incubation with 2.5 mM KCN and 100 μM H_2O_2 for 0.5 h (b, II and c, II), 2.5 h (b, III), and 8 h (d, II). Single arrows (b) indicate EC nuclei, and doubled arrows indicate GC nuclei.

the light. $FeSO_4$ and ascorbate added separately on the background of CN^- were even more effective than their combination. EGTA, a bivalent cation chelator, induced *per se* the destruction of GC nuclei and enhanced the effect of CN^- (Fig. 3b).

Effect of electron acceptors on the destruction of GC nuclei induced by CN^- and H_2O_2. Figure 4 (line *1*) illustrates the O_2 consumption by pea leaf slices induced by oxidation of intracellular substrates (endogenous respiration) in the dark. When the light was switched on, a release of O_2 was observed in association with electron transfer in chloroplasts from H_2O to $NADP^+$. A lag-phase in photosynthetic O_2 evolution is due to the regulatory alterations in systems of electron transport; it can be

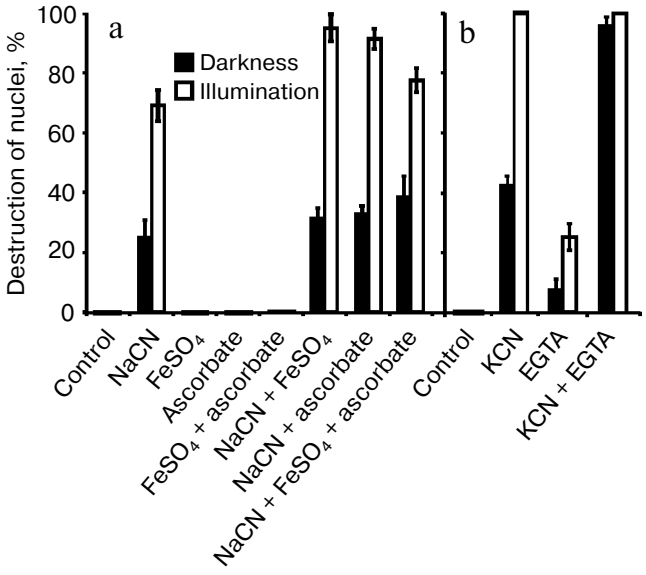

Fig. 3. Effect of $FeSO_4$ + ascorbate (a) and EGTA (b) on the CN^--induced destruction of GC nuclei in pea leaf epidermis peels in the darkness and in the light. Epidermal peels were infiltrated with 10 μM $FeSO_4$, 10 mM Na-ascorbate, and 2 mM EGTA followed by incubation for 15 min in darkness, then 2.5 mM NaCN or KCN were added and, after repeated infiltration, the peels were subjected to a second incubation for 18 h (a) or 22 h (b) in darkness or in the light.

observed in isolated chloroplasts as well [32], and its nature was considered earlier [33, 34]. In intact leaves, the lag-phase apparently reflects also the interaction between chloroplasts and mitochondria. KCN inhibited the O_2 evolution (Fig. 4, line *1*). CN^- induced Rubisco inactivation [5] and thus inhibited $NADP^+$ regeneration from NADPH. The deficit of $NADP^+$ resulted in inhibition of CO_2-dependent photosynthetic O_2 evolution replaced with O_2 uptake in the light. This process, with the rate independent on light switching off, is due to the mitochondrial respiration with an alternative oxidase (ubiquinol oxidase) resistant to CN^- (see for review [35]). Its rate was decreased by addition of *p*-benzoquinone (BQ), a membrane-penetrating electron acceptor from mitochondrial ubiquinol. Ferricyanide, an electron acceptor not penetrating membranes, was added to the incubation mixture to maintain the level of oxidized BQ. Restored O_2 evolution was observed after following illumination: the Hill reaction, i.e., the photoinduced electron transfer from H_2O to the added BQ, was switched on. The process was inhibited by DCMU blocking the electron transfer between plastoquinones Q_A and Q_B in Photosystem II. The O_2 uptake insensitive to the blackout or the light switching on (intervals *a* and *b*) could be inhibited by the propyl gallate, an inhibitor of alternative oxidase. Results similar to those obtained with leaf slices (Fig. 4, line *1*) were obtained with isolated epidermal peels (data not shown).

As one can see from Fig. 4 (lines *2* and *3*), the respiration of leaf slices (LS) is inhibited by CN^- and propyl gallate. Both menadione and methyl viologen induce light-dependent electron transfer: being reduced by the components of photosynthetic chain, menadione and methyl viologen are spontaneously oxidized by O_2. The process was stopped in the dark. Catalase induced O_2 release, thus suggesting H_2O_2 formation. Menadione and methyl viologen enhanced by 1.5-1.7 times the LS respiration via their interaction with respiratory chain (Fig. 4, lines *4* and *5*); the respiration was inhibited by the subsequent addition of catalase.

Electron acceptors maintaining the Hill reaction in chloroplasts inhibited the CN^--induced destruction of GC nuclei [7, 8]. Table 1 shows that the added electron acceptors per se did not influence the state of GC nuclei, but effectively prevented their destruction induced by $CN^- + H_2O_2$ in the light. The CN^--induced destruction of EC nuclei was virtually unchanged when electron acceptors, such as methyl viologen, *p*-benzoquinone, and menadione, were added.

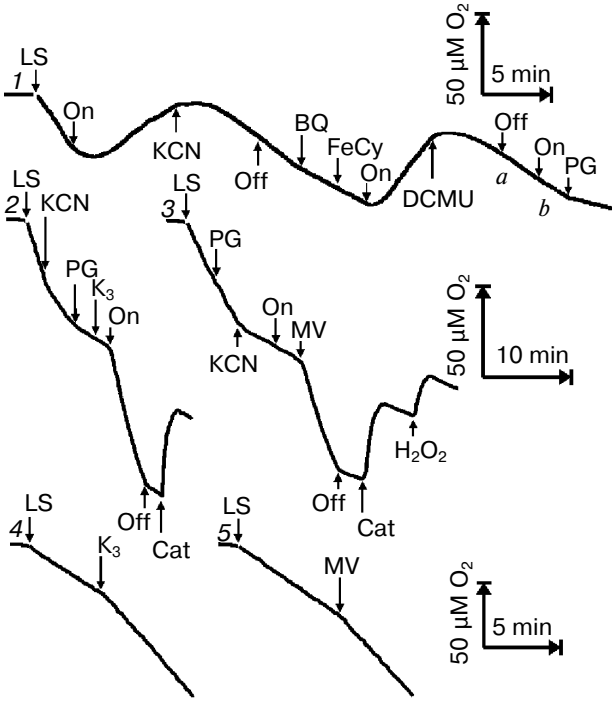

Fig. 4. Consumption and evolution of O_2 by pea leaf slices (LS). Incubation medium: 10 mM HEPES-NaOH, pH 7.0, 25 mM KCl, and 10 mg/ml LS with the chlorophyll content of 45.3 μg/ml. Additives: 2.5 mM KCN, 100 μM *p*-benzoquinone (BQ), 3 mM potassium ferricyanide (FeCy), 10 μM DCMU, 10 μM propyl gallate (PG), 100 μM menadione (vitamin K_3), 5 mM methyl viologen (MV), 50 μM H_2O_2, and 2 U/ml catalase (Cat). On and Off, switching on and switching off the light. The initial respiratory rates and photosynthetic O_2 evolution (in absence of additives, such as in the experiment *1*) were: 10.7-11.2 and 26.7-27.3 μmoles O_2/mg chlorophyll per h.

Table 1. Effect of electron acceptors on the (KCN + H_2O_2)-induced destruction of GC nuclei and KCN-induced destruction of EC nuclei in pea leaf epidermis peels

Additives	Destruction of nuclei, %
GC, light	
—	0
Electron acceptors	0
KCN + H_2O_2	97.0 ± 2.2
» + *p*-benzoquinone	0
» + diaminodurol	1.8 ± 1.4
» + 2,6-dichlorophenol-indophenol	13.7 ± 2.3
» + menadione	24.3 ± 6.5
» + methyl viologen*	0
» + TMPD	0.2 ± 0.4
EC, darkness	
—	22.6 ± 5.4
Methyl viologen	15.2 ± 5.0
Menadione	23.6 ± 7.3
p-Benzoquinone	20.3 ± 7.7
KCN	90.7 ± 4.2
» + methyl viologen*	89.1 ± 9.6
» + menadione	91.8 ± 8.3
» + *p*-benzoquinone	78.2 ± 5.9

Note: Epidermal peels after infiltration with electron acceptors at 100 μM or 5 mM (*) concentrations were incubated in the dark for 30 min, then, for GC, 2.5 mM KCN + 100 μM H_2O_2 were added, and the peels were incubated for 15 h in the light, and for EC the peels were supplied with 2.5 mM KCN with following incubation for 1 h in the dark, fixed, and examined under the microscope.

Effect of DCMU and DNP-INT on the destruction of GC nuclei induced by CN^- and CN^- + H_2O_2. In accordance with the data obtained earlier [7, 8], the CN^--induced apoptosis in GC was prevented by DCMU and DNP-INT (Fig. 5a). Their effect on GC was abolished or drastically decreased by the treatment of the epidermis with CN^- + H_2O_2 combination. DCMU and DNP-INT did not prevent the CN^--induced destruction of EC nuclei (Fig. 5b).

Effect of CN^- and H_2O_2 on the efficiency of DCMU as an inhibitor of electron transfer in chloroplasts. Pea leaf slices (LS) were pre-incubated with CN^-, H_2O_2, or CN^- + H_2O_2 in the light and washed with distilled water, and the level of DCMU-induced inhibition of the Hill reaction with BQ + FeCy as electron acceptor pair was measured as in the experiment shown in the Fig. 4, interval *a* (O_2 uptake in the dark due to the action of alternative oxidase) and interval *b* (alternative oxidase action and Photosystem II activity, if it is not completely inhibited by DCMU). The data given in the Table 2 demonstrate that in the control LS sample the consumption rates within the intervals *a* and *b* are equal, and their ratio is 1.0. Their ratio increased to 1.2, when LS were incubated with H_2O_2: DCMU did not inhibit completely the Hill reaction. Much greater increase in the ratio, up to 1.6, was observed when LS were incubated with H_2O_2 + CN^-. Thus, H_2O_2 and even more H_2O_2 + CN^- decreased efficiency of DCMU.

CN^--induced destruction of GC nuclei under conditions when only Photosystem I is active. Inhibition of Photosystem II in chloroplasts with DCMU switches the photosynthetic chain from the non-cyclic regimen of electron transfer from H_2O to $NADP^+$ to the cyclic electron transfer regimen involving Photosystem I and cytochrome b_6f complex. N,N,N′,N′-Tetramethyl-*p*-phenylenediamine (TMPD) + ascorbate, an electron donor pair for Photosystem I, switches the electron transfer chain to the regimen of non-cyclic electron transfer from TMPD to $NADP^+$ dependent on Photosystem I. CN^--induced destruction of GC nuclei in the light was significantly decreased by DCMU or DCMU + TMPD + ascorbate (Fig. 5c).

Effects of inhibitors of plasma membrane-associated NAD(P)H-oxidase. The effect of quinacrine and diphenyleneiodonium (DPI), the inhibitors of plasma membrane-associated NAD(P)H-oxidase [36-39], was examined in the final experiments. Quinacrine at con-

Table 2. Efficiency of DCMU as an inhibitor of Photosystem II in the Hill reaction with BQ + FeCy in pea leaf slices (LS) treated with H_2O_2 and CN^- + H_2O_2

LS treatment mode	Ratio of alteration rates of [O_2] in dark and in light (*b*/*a*, as in Fig. 4)
Control, without treatment	1.0
H_2O_2	1.2
H_2O_2 + KCN	1.6

Note: LS were pre-incubated for 30 min in the light with 1 mM H_2O_2 or with 1 mM H_2O_2 + 2.5 mM KCN, washed with distilled water, and placed in the oximetric cell; then 2.5 mM KCN, 100 μM BQ, and 3 mM FeCy were added with following incubation for 3 min in the dark and measurement of O_2 evolution rates in Hill reaction, which were similar and comprised 40-41 μmoles O_2/mg chlorophyll per h in control variant and LS treated with H_2O_2 or H_2O_2 + KCN. Then 10 μM DCMU was added followed by incubated for 3 min in the dark and measurement of the rates of [O_2] alterations in the dark (interval *a*, as on Fig. 4) and in the light (interval *b*, as on Fig. 4).

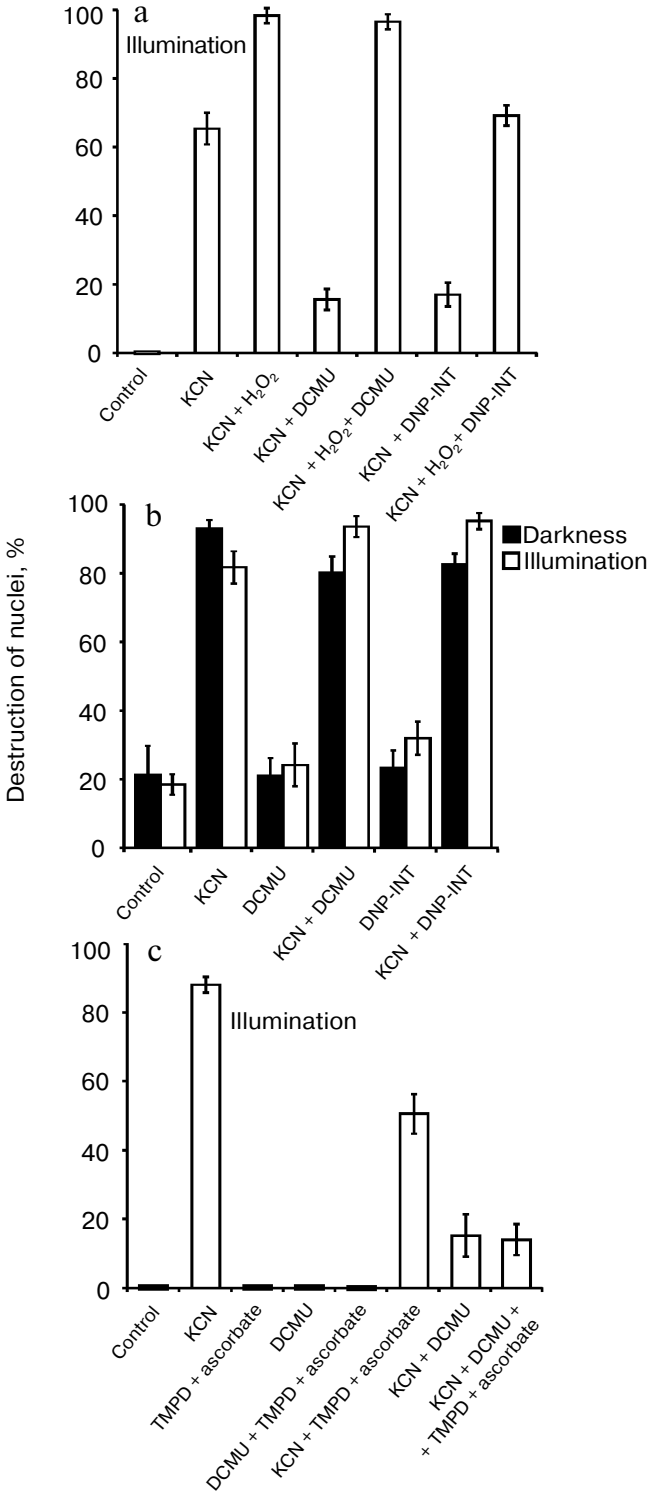

Fig. 5. Effect of DCMU and DNP-INT on CN^-- and (CN^- + H_2O_2)-induced destruction of GC nuclei (a) and CN^--induced destruction of EC nuclei (b) and effect of DCMU and its combination with N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate on CN^--induced destruction of GC nuclei (c) in pea leaf epidermis. Epidermal peels were subjected to infiltration with 10 μM DCMU or 10 μM DNP-INT followed by incubation for 30 min in the dark; then the peels were infiltrated with 2.5 mM KCN ± 100 μM H_2O_2, TMPD, and ascorbate followed by incubation for 13 (a), 1 (b), or 21 h (c).

centrations up to 100 μM and DPI (up to 100-200 μM) did not influence the mitochondrial respiration and photosynthetic O_2 evolution by chloroplasts in LS, but prevented CN^--induced destruction of GC nuclei (Figs. 6 and 7) in leaf epidermis peels. Quinacrine did not influence the CN^--induced destruction of EC nuclei (Fig. 6d).

Figure 8a illustrates the H_2O_2-dependent formation of fluorescent dichlorofluorescein (DCF) in epidermal peels. Non-fluorescent dichlorofluorescin (DCFH) diacetate added to the peel incubation medium diffuses across the plasma membrane; thereafter it is hydrolyzed by intracellular esterases to non-fluorescent DCFH and accumulated in the cells wherein it is oxidized to fluorescent DCF. DCFH is oxidized to DCF by H_2O_2, either enzymatically by peroxidase or non-enzymatically in the presence of Fe^{2+} [40]. DCFH is also oxidized by $OH^{\cdot}$ and, $CO_3^{\cdot-}$ and more slowly by $NO_2^{\cdot}$, but not $O_2^{\cdot-}$ [41]. Catalase inhibited and prevented H_2O_2-dependent enhancement of DCF fluorescence (Fig. 8a). Menadione also induced the fluorescence enhancement, although with slower rate than H_2O_2 (Fig. 8b). The DCF fluorescence growth induced by menadione was inhibited by nitroblue tetrazolium (NBT) (Fig. 8c) oxidizing $O_2^{\cdot-}$ [42] and thus preventing the formation of H_2O_2. Quinacrine inhibited and prevented H_2O_2- and menadione-induced DCF response (Fig. 8, d-f).

DISCUSSION

CN^--induced death of GC and generation of ROS in Photosystem I. Incubation of isolated chloroplasts with CN^- resulted in fragmentation of the large subunit of Rubisco and thus disturbance of the enzyme function [5]. The enzyme also became inactivated when H_2O_2 was added to chloroplasts incubated in the light, but not in the dark [5]. Half maximum inhibition of CO_2 fixation happened at H_2O_2 concentration of 10 μM [43]. It has been concluded that CN^--induced degradation of Rubisco in chloroplasts is due to a combination of two factors: generation of H_2O_2 and accumulation of photosynthetic chain components in reduced form, most probably, ferredoxin and FeS-centers, in Photosystem I [5]. The conclusion that H_2O_2 alone is insufficient for Rubisco degradation agrees with the data on stability of isolated Rubisco to H_2O_2: elevation of H_2O_2 concentration up to 10 mM does not influence the isolated enzyme [44].

CN^--induced disturbance of CO_2 fixation in chloroplasts results in the transition of the electron acceptor branch of Photosystem I into the reduced state. As a result of auto-oxidation of these components, mainly FeS-centers F_X, F_A, and F_B, by oxygen $O_2^{\cdot-}$ is generated in chloroplasts [4], which can be transformed into H_2O_2 by superoxide dismutase, although partially inhibited by

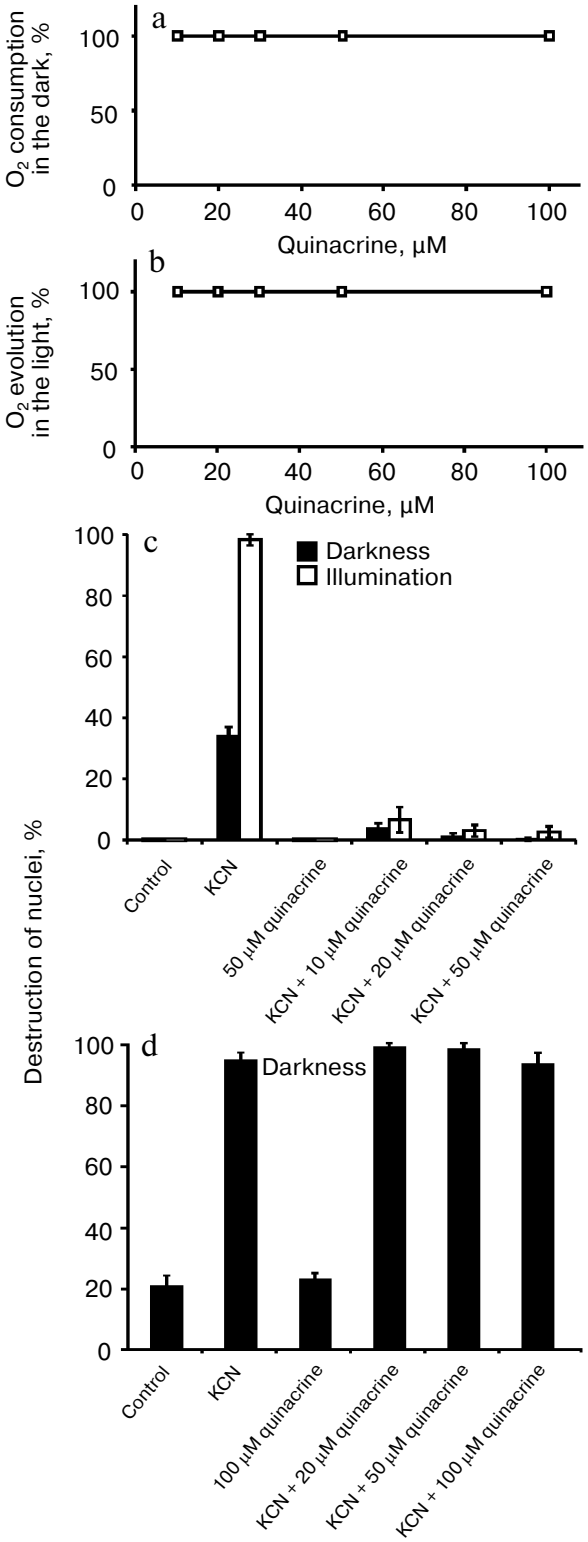

Fig. 6. Effect of quinacrine on respiration (a), photosynthetic O_2 evolution (b) in leaf slices, and CN^--induced destruction of GC (c) and EC (d) nuclei in pea leaf epidermis. Epidermal peels were infiltrated with quinacrine followed by incubation for 30 min; thereafter they were infiltrated with 2.5 mM KCN and incubated in the dark or in the light for 22 h in experiment (c) and for 1 h in experiment (d). Other conditions were the same as in Fig. 4.

cyanide, as well as can be converted into H_2O_2 via non-enzymatic disproportionation. If $O_2^{\cdot-}$ and H_2O_2 generated by Photosystem I are ineffectively removed (due to the presence of cyanide, an inhibitor of Cu,Zn-superoxide dismutase, catalase, and ascorbate peroxidase), the hydroxyl radical $OH^{\cdot}$, the potent oxidant (see a review [45] about ferrous and cupric ions in radical reactions), is formed as a result of their interaction with trace amounts of free ferrous (Fenton's reaction and reaction of Haber–Weiss), copper, or manganese ions. These metal cations can be introduced, for instance, with water (external sources). Existence of internal sources is possi-

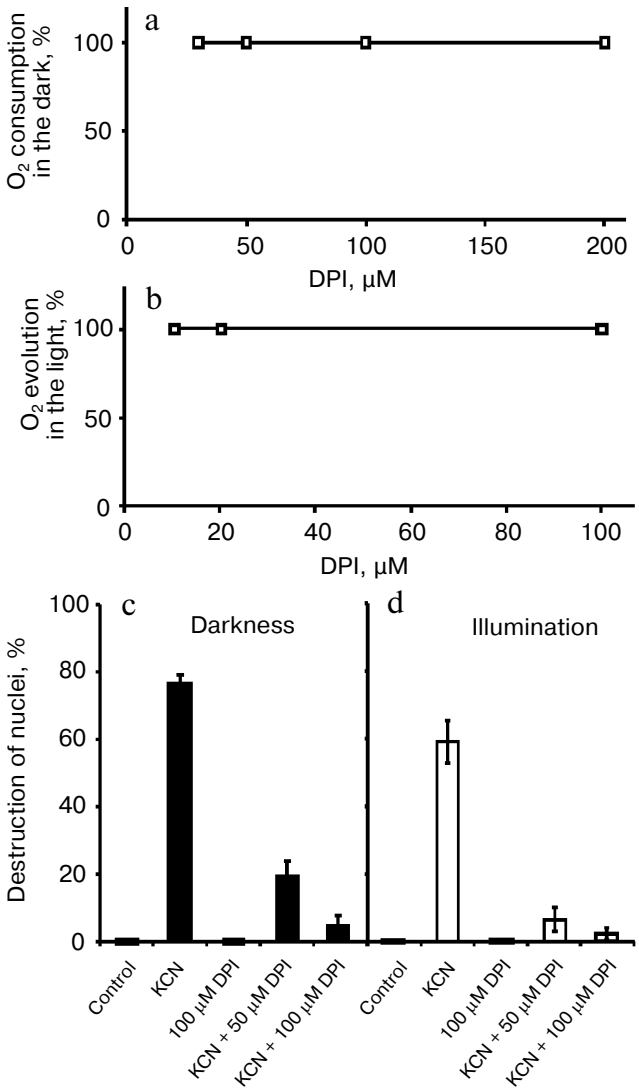

Fig. 7. Effect of diphenyleneiodonium (DPI) on respiration (a) and photosynthetic O_2 evolution (b) in leaf slices and on CN^--induced destruction of GC nuclei in the dark (c) and in the light (d) in pea leaf epidermis. The epidermal peels were infiltrated with DPI followed by incubation for 30 min; thereafter they were infiltrated with 2.5 mM KCN and incubated for 23 h in experiment (c) and for 15 h in experiment (d). Other conditions were the same as in Fig. 4.

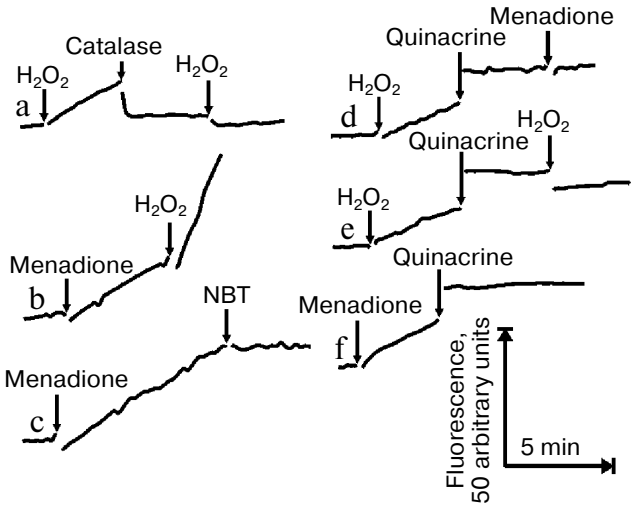

Fig. 8. DCF formation from DCFH diacetate added to leaf epidermis peels. Additives: 30 μM H_2O_2, 2 U/ml catalase, 100 μM menadione, 200 μM NBT, and 50 μM quinacrine.

ble as well. In particular, H_2O_2 induced manganese release from oxygen-evolving complex of cyanobacteria [46]. $OH^{\cdot}$ radicals can be formed also from the interaction between H_2O_2 and FeS-centers of Photosystem I [47]. The Fenton reaction with participation of Fe^{2+} in biomolecules flows with higher rates than with the participation of free hexa-aquo-Fe^{2+} [48].

Are these above-described processes leading to $OH^{\cdot}$ generation the cause of stimulation of CN^--induced apoptosis of GC under illumination? The answer to this question must apparently be negative, because, on one hand, any clear effect of Fe^{2+} and Fe^{2+} + ascorbate on CN^--induced destruction of nuclei was absent (Fig. 3a). Formation of the complex salt ferrocyanide resulting from the interaction of Fe^{2+} and CN^- without intermediate Fe^{2+}-cyanide formation should be taken into account. The formation of ferrocyanide would inhibit the Fenton reaction [49]. On the other hand, EGTA added for chelating of free transient valency cations, which can be present in the analyzed samples enhanced the effect of CN^- instead of anticipated alleviation (Fig. 3b). $O_2^{\cdot-}$ and H_2O_2 must be generated on the background of CN^- with TMPD and ascorbate, the electron donor pair for chloroplast Photosystem I. If the light stimulation of CN^--induced destruction of GC nuclei would be associated with $OH^{\cdot}$-generating activity of FeS-centers of the electron acceptor branch of Photosystem I (Photosystem II is blocked by DCMU), TMPD + ascorbate should not inhibit, but enhance the effect of CN^- (Fig. 5c).

Thus, the light stimulation of the CN^--induced PCD of GC cannot be due to the disturbance of Rubisco function and $OH^{\cdot}$ generation induced by this disturbance with participation of free transient valency metals in reactions of Fenton or Haber–Weiss types, as well as with participation of FeS-centers of electron acceptor branch of Photosystem I.

CN^--induced GC death and ROS generation in chloroplasts and mitochondria. Electron acceptors supporting the Hill reaction in chloroplasts and capable of interacting with mitochondrial respiratory chain prevent CN^--induced apoptosis of GC, but not of EC [7, 8]. The Hill reagents have the same effect on GC death in the presence of CN^- + H_2O_2 (Table 1). Among the tested electron acceptors were menadione and methyl viologen, which are widely used as inducers of extracellular ROS formation. Methyl viologen (paraquat), a potent herbicide, is reduced by Photosystem I, principally by FeS-center F_B [50]. Linear electron flow switched on by Photosystem I is intercepted by added methyl viologen almost by 100% [4]. Pseudo-cyclic electron transfer to O_2 ("water-water" cycle) comprises ~30% of total linear electron flow in the absence of added electron acceptors in various C_3- and C_4-plants and algae [4]. Methyl viologen has also multiple effects on mitochondria via its interaction with the respiratory chain [51]. Menadione is reduced by Photosystem II, cytochrome b_6f complex, and Photosystem I in chloroplasts [52, 53], as well as by mitochondrial NADH:ubiquinone oxidoreductase [54] and cytochrome bc_1 complex [55]. The products of methyl viologen and menadione reduction are spontaneously oxidized by O_2 to form $O_2^{\cdot-}$, and $O_2^{\cdot-}$ transforms into H_2O_2. Menadiol is also oxidized by O_2 to form H_2O_2 [54, 56].

Oximetry data show that both menadione and methyl viologen induce H_2O_2 formation in illuminated LS (Fig. 4): addition of catalase results in O_2 release. In spite of intensive H_2O_2 formation in chloroplasts, menadione and methyl viologen do not enhance, but virtually nullify the CN^-- [7, 8] and CN^- + H_2O_2-induced apoptosis of GC (Table 1). Generation of H_2O_2 in mitochondria (Fig. 4) also does not enhance the death of GC and EC (Table 1).

Thus, addition of H_2O_2 accelerates PCD, whereas endogenous H_2O_2 generated by chloroplasts and mitochondria does not induce this effect.

Role of plasma membrane-associated NAD(P)H-oxidase in PCD. CN^--induced death of GC, but not EC, was inhibited by quinacrine (Fig. 6) and DPI (Fig. 7), the inhibitors of flavin dehydrogenases at concentrations, which do not influence mitochondrial respiration and photosynthetic O_2 evolution by chloroplasts in LS. Quinacrine inhibited H_2O_2-dependent oxidation of nonfluorescent DCFH to fluorescent DCF as a response to H_2O_2 or menadione addition (Fig. 8).

These results indicate that NAD(P)H-oxidase of plasma membrane is a source of ROS in CN^--induced death of GC. To judge by the data on inhibitory effect of quinacrine (Fig. 8), H_2O_2 exogenously added or generated in the presence of menadione, activates NAD(P)H-oxidase of plasma membrane. The effect of ROS-induced

ROS production is known in mitochondria of cardiomyocytes [57]. Stimulation of NAD(P)H-oxidase by H_2O_2 was observed in non-phagocytic cells originated from vessels [58].

Not every ROS source is suitable for realization of PCD, which may be due to the compartmentalization of the cell. ROS formed in chloroplasts and mitochondria apparently meet obstacles on the way of their targeting to the sites of their realization in programmed cell death: $O_2^{\cdot-}$ by itself cannot penetrate membranes, H_2O_2 is subjected to decomposition by catalases and peroxidases. Therefore, NAD(P)H-oxidase of plasma membrane proves to be an effective source of ROS. Exogenously added H_2O_2 possesses stimulatory effect on PCD when the source used in PCD insufficiently generates ROS.

Nevertheless, chloroplasts play an important role in PCD. Inhibition of Photosystem II by DCMU or b_6f complex by DNP-INT and stigmatellin prevents PCD in GC [7, 8]. The data obtained with pea mutants [9] have shown that light stimulation of CN^--induced PCD of GC depends on Photosystem II. These effects can be due to the participation of protein kinase regulated by the photosynthetic chain of chloroplasts in PCD [7, 8].

H_2O_2-induced decrease in DCMU and DNP-INT effects on programmed death of GC. According to data published earlier [7, 8], DCMU and DNP-INT inhibited CN^--induced death of GC (Fig. 5). H_2O_2 decreased effects of both inhibitors.

H_2O_2 and largely H_2O_2 + CN^- decreased the inhibitory effect of DCMU on the photosynthetic O_2 evolution (Table 2). H_2O_2 also decreased the inhibitory effect of DCMU on O_2 evolution by Photosystem II of catalase-deficient sub-chloroplast particles in the light [59]: H_2O_2 induced disturbance in Photosystem II due to the decrease in DCMU ability to react with the secondary plastoquinone binding site Q_B. It has been concluded that heme catalase of Photosystem II plays an important role in defense of O_2-evolving complex from H_2O_2 [59].

Possibly, the ability of DNP-INT to bind cytochrome b_6f complex in chloroplasts becomes altered in a similar way. However, other possibilities cannot be excluded, in particular, changed mode of b_6f complex action. Various bypasses of the reaction on the site *o* of bc_1 cytochrome complex have been described [60].

REFERENCES

1. Samuilov, V. D., Lagunova, E. M., Beshta, O. E., and Kitashov, A. V. (2000) *Biochemistry (Moscow)*, **65**, 696-702.
2. Wang, H., Li, J., Bostock, R. M., and Gilchrist, D. G. (1996) *Plant Cell*, **8**, 375-391.
3. Ryerson, D. E., and Heath, M. C. (1996) *Plant Cell*, **8**, 393-402.
4. Asada, K. (2000) *Phil. Trans. R. Soc. Lond. B*, **355**, 1419-1431.
5. Ishida, H., Shimizu, S., Makino, A., and Mae, T. (1998) *Planta*, **204**, 305-309.
6. Bakeeva, L. E., Dzyubinskaya, E. V., and Samuilov, V. D. (2005) *Biochemistry (Moscow)*, **70**, 972-979.
7. Samuilov, V. D., Lagunova, E. M., Dzyubinskaya, E. V., Izyumov, D. S., Kiselevsky, D. B., and Makarova, Ya. V. (2002) *Biochemistry (Moscow)*, **67**, 627-634.
8. Samuilov, V. D., Lagunova, E. M., Kiselevsky, D. B., Dzyubinskaya, E. V., Makarova, Ya. V., and Gusev, M. V. (2003) *Biosci. Rep.*, **23**, 103-117.
9. Samuilov, V. D., Lagunova, E. M., Gostimsky, S. A., Timofeev, K. N., and Gusev, M. V. (2003) *Biochemistry (Moscow)*, **68**, 912-917.
10. Rusterucci, C., Aviv, D. H., Holt, B. F., Dangl, J. L., and Parker, J. E. (2001) *Plant Cell*, **13**, 2211-2224.
11. Brodersen, P., Petersen, M., Pike, H. M., Olszak, B., Skov, S., Odum, N., Jorgensen, L. B., Brown, R. E., and Mundy, J. (2002) *Genes Dev.*, **16**, 490-502.
12. Gray, J., Janick-Buckner, B., Close, P. S., and Johal, G. S. (2002) *Plant Physiol.*, **130**, 1894-1907.
13. Lorrain, S., Vailleau, F., Balaque, C., and Roby, D. (2003) *Trends Plant Sci.*, **8**, 263-271.
14. Asai, T., Stone, J. M., Heard, J. E., Kovtun, Y., Yorgey, P., Sheen, Y., and Ausubel, F. M. (2000) *Plant Cell*, **12**, 1823-1836.
15. Danon, A., Rotari, V. I., Gordon, A., Mailhac, N., and Gallois, P. (2004) *J. Biol. Chem.*, **279**, 779-787.
16. Seo, S., Okamoto, M., Iwai, T., Iwano, M., Fukui, K., Isogai, A., Nakajima, N., and Ohashi, Y. (2000) *Plant Cell*, **12**, 917-932.
17. Van Loo, G., Saelens, X., van Gurp, M., MacFarlane, M., Martin, S. J., and Vandenabeele, P. (2002) *Cell Death Differ.*, **9**, 1031-1042.
18. Lam, E., Kato, N., and Lawton, M. (2001) *Nature*, **411**, 848-853.
19. Lam, E. (2004) *Nature Rev. Mol. Cell Biol.*, **5**, 305-315.
20. Balk, J., Chew, S. K., Leaver, C. J., and McCabe, P. F. (2003) *Plant J.*, **34**, 573-583.
21. Vranova, E., Inze, D., and van Breusegem, F. (2002) *J. Exp. Bot.*, **53**, 1227-1236.
22. Wojtaszek, P. (1997) *Biochem. J.*, **322**, 681-692.
23. Neill, S. J., Desikan, R., Clarke, A., Hurst, R. D., and Hancock, J. T. (2002) *J. Exp. Bot.*, **53**, 1237-1247.
24. Braidot, E., Petrussa, E., Vianello, A., and Macri, F. (1999) *FEBS Lett.*, **451**, 347-350.
25. Tiwari, B. S., Belenghi, B., and Levine, A. (2002) *Plant Physiol.*, **128**, 1271-1281.
26. Arnon, D. I. (1949) *Plant Physiol.*, **24**, 1-15.
27. Trotta, E., Del Grosso, N., Erba, M., Melino, S., Cicero, D., and Paci, M. (2003) *Eur. J. Biochem.*, **270**, 4755-4761.
28. Halliwell, B. (1996) *Free Rad. Res.*, **25**, 439-454.
29. Podmore, I. D., Griffiths, H. R., Herbert, K. E., Mistry, N., Mistry, P., and Lunec, J. (1998) *Nature*, **392**, 559.
30. Rose, R. C., Choi, J. L., and Bode, A. M. (1992) *Life Sci.*, **50**, 1543-1549.
31. Song, J. H., Shin, S. H., Wang, W., and Ross, G. M. (2001) *Exp. Neurol.*, **169**, 425-437.
32. Allen, J. F. (1984) *FEBS Lett.*, **166**, 237-244.
33. Takahama, U., Shimizu-Takahama, M., and Heber, U. (1981) *Biochim. Biophys. Acta*, **637**, 530-539.
34. Samuilov, V. D., and Fedorenko, T. A. (1999) *Biochemistry (Moscow)*, **64**, 610-619.
35. Affourtit, C., Albury, M. S., Crichton, P. G., and Moore, A. L. (2002) *FEBS Lett.*, **510**, 121-126.

36. Van Gestelen, P., Asard, H., and Caubergs, R. J. (1997) *Plant Physiol.*, **115**, 543-550.
37. Papadakis, A. K., and Roubelakis-Angelakis, K. A. (1999) *Plant Physiol.*, **121**, 197-205.
38. Frahry, G., and Schopfer, P. (2001) *Planta*, **212**, 175-183.
39. Dat, J. F., Pellinen, R., Beeckman, T., van De Cotte, B., Langebartels, C., Kangasjarvi, J., Inze, D., and van Breusegem, F. (2003) *Plant J.*, **33**, 621-632.
40. LeBel, C. P., Ischiropoulos, H., and Bondy, S. C. (1992) *Chem. Res. Toxicol.*, **5**, 227-231.
41. Wrona, M., Patel, K., and Wardman, P. (2005) *Free Rad. Biol. Med.*, **38**, 262-270.
42. Auclair, C., and Voisin, E. (1985) in *CRC Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., ed.) CRC Press, Inc., Boca Raton, Florida, pp. 123-132.
43. Kaiser, W. M. (1976) *Biochim. Biophys. Acta*, **440**, 476-482.
44. Li, S., Lu, W., Li, G.-F., Gong, Y.-D., Zhao, N.-M., Zhang, R.-X., and Zhou, H.-M. (2004) *Biochemistry (Moscow)*, **69**, 1136-1142.
45. Koppenol, W. H. (1994) *Free Radical Damage and Its Control* (Rice-Evans, C. A., and Burdon, R. H., eds.) Elsevier Sci. B. V., pp. 3-24.
46. Samuilov, V. D., Timofeev, K. N., Sinitsyn, S. V., and Bezryadnov, D. V. (2004) *Biochemistry (Moscow)*, **69**, 926-933.
47. Jakob, B., and Heber, U. (1996) *Plant Cell Physiol.*, **37**, 629-635.
48. Wardman, P., and Candeias, L. P. (1996) *Radiat. Res.*, **145**, 523-531.
49. Ilan, Y. A., Czapski, G., and Meisel, D. (1976) *Biochim. Biophys. Acta*, **430**, 209-224.
50. Fujii, T., Yokoyama, E., Inoue, K., and Sakurai, H. (1990) *Biochim. Biophys. Acta*, **1015**, 41-48.
51. Palmeira, C. M., Moreno, A. J., and Madeira, V. M. C. (1995) *Biochim. Biophys. Acta*, **1229**, 187-192.
52. Hauska, G. (1977) in *Encyclopedia of Plant Physiology* (Trebst, A., and Avron, M., eds.) Vol. 5, Springer-Verlag, Berlin, pp. 253-265.
53. Samuilov, V. D., Barsky, E. L., and Kitashov, A. V. (1997) *Biochemistry (Moscow)*, **62**, 909-913.
54. Cadenas, E., Boveris, A., Ragan, C. I., and Stoppani, A. O. (1977) *Arch. Biochem. Biophys.*, **180**, 248-257.
55. Kolesova, G. M., Karnaukhova, L. V., and Yaguzhinskii, L. S. (1991) *Biokhimiya*, **56**, 1779-1786.
56. Yamashoji, S., Ikeda, T., and Yamashoji, K. (1991) *Biochim. Biophys. Acta*, **1059**, 99-105.
57. Zorov, D. B., Filurm, C. R., Klotz, L. O., Zweier, J. L., and Sollott, S. J. (2000) *J. Exp. Med.*, **192**, 1001-1014.
58. Li, W.-G., Miller, F. J., Zhang, H. J., Spitz, D. R., Oberley, L. W., and Weintraub, N. L. (2001) *J. Biol. Chem.*, **276**, 29251-29256.
59. Sheptovitsky, Y. G., and Brudvig, G. W. (1998) *Biochemistry*, **37**, 5052-5059.
60. Muller, F., Crofts, A. R., and Kramer, D. M. (2002) *Biochemistry*, **41**, 7866-7874.