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František ŠERŠEŇ[1*] and Katarína KRÁĽOVÁ[1]

ACTION OF SOME ORGANOMERCURY COMPOUNDS ON PHOTOSYNTHESIS IN SPINACH CHLOROPLASTS

WPŁYW NIEKTÓRYCH ZWIĄZKÓW RTĘCI NA FOTOSYNTEZĘ W CHLOROPLASTACH SZPINAKU

Abstract: The effects of five organomercury compounds (methylmercuric chloride, phenylmercuric acetate, phenylmercuric borate, phenylmercuric citrate and diphenylmercury) on photosynthetic electron transport (PET) in spinach chloroplasts were investigated. The IC_{50} values of organomercury compounds related to PET inhibition in spinach chloroplasts varied in the range from 468 μmol dm^{-3} to 942 μmol dm^{-3} and were approximately by one order higher than the corresponding value determined for $HgCl_2$ applied also in DMSO solution (IC_{50} = 58 μmol dm^{-3}). Due to extremely low aqueous solubility of diphenylmercury, the corresponding IC_{50} value could not be determined. Using EPR spectroscopy as probable sites of action of organomercury compounds in photosynthetic apparatus ferredoxin on the acceptor side of PS 1 and the quinone electron acceptors Q_A or Q_B on the reducing side of PS 2 were suggested.

Keywords: EPR spectroscopy, organomercurials, photosynthetic electron transport

Introduction

Mercury belongs among the nitrogen/sulphur-seeking metals categorized into class B, which are characterized by a high affinity to the *N*- and *S*-donor ligands occurring in many biomacromolecules [1]. Consequently, Hg^{2+} ions have greater affinity towards sulphur containing amino acids than towards other amino acids. They attack the –SH bonds and the binding preference of Hg^{2+} for sulfhydryl and thioether groups at catalytically active centres in enzymes provide the biochemical basis for much of mercury toxicity. The formation constant for Hg^{2+} and the anionic form of a sulfhydryl group $R–S^-$ is $\geq 10^{10}$- times higher than that determined for the carboxyl or amino groups [2].

Mercury exhibits many adverse effects on plants, *eg* it inhibits seed germination and seedling growth of higher plants [3, 4] and toxic effects of mercury are reflected also by decrease of the total protein content [5], reduction of water content, chlorophyll concentration and nutrient translocation in plants [6-8] as well as by reduced photosynthesis and transpiration [9]. In *Euglena gracilis*, $HgCl_2$ inhibited synthesis of chlorophyll [10]. At

[1] Faculty of Natural Sciences, Comenius University in Bratislava, Mlynská dolina CH2, SK-84215 Bratislava, Slovak Republic, phone: +42160296412
[*]Corresponding author: sersen@fns.uniba.sk

sublethal concentrations mercury displaced the Mg^{2+} ion of the tetrapyrrol ring, causing imbalances in protochlorophyll formation as well as affecting both chlorophyll synthesis and degradation [11].

Mercury ions can form more or less stable complexes with many biomolecules, what is reflected also in their effectiveness related to the inhibition of photosynthetic processes in algae and plant chloroplasts. Hg^{2+} ions are able to interact with some components of the photosynthetic electron transport (PET) chain, *eg* with plastocyanin on the donor side of photosystem (PS) 1 [12, 13], with ferredoxin [14] or with F_B-iron-sulphur cluster [15] which are localized on the acceptor side of PS 1. Further possible sites of Hg^{2+} ions action are situated in the oxygen evolving complex [14, 16, 17], in the core of PS 2 [18] and between Q_A and Q_B on the acceptor side of PS 2 [19].

On the other hand, there is less data in the literature concerning the effects of organomercury compounds on photosynthesis. Phenylmercuric acetate inhibited Hill activity, ferredoxin-NADP oxidoreductase and photophosphorylation [20, 21]. Godbold and Huttermann [22] found that photosynthesis was inhibited to a lower extent in spruce plants exposed to $HgCl_2$ than in those exposed to methylmercuric chloride. Singh and Singh [23] observed a synergetic effect at the application of CH_3HgCl in combination with $HgCl_2$. Phenylmercuric acetate is known to be a pesticide and is used as an ingredient in Agrosan preparation [24]. Phenylmercuric borate is known as a local external antiseptic agent.

The toxic effect of methylmercury cation ($MeHg^+$) on the photosynthetic activity of *Chlorella vulgaris* was shown to increase under high illumination and unfavourable low temperature. Increased toxic action of $MeHg^+$ resulted from the decreased capacity of PS 2 for reparation [25]. Kukarskikh et al [26] determined chlorophyll fluorescence parameters in green microalgae *Chlamydomonas reinhardtii* and found that after $MeHg^+$ treatment algal cells indicated damage on the donor side of PS 2 and impairment of the electron transfer from Q_A to Q_B occurred. A disturbance of the electron transfer between photosystems was also confirmed by an increase in the steady-state level of P700 photooxidation. The above results demonstrated that $MeHg^+$ treatment damaged the PET at several sites, although the inhibitory effect of $MeHg^+$ on photosynthetic processes of algae was much stronger than the effect of $HgCl_2$. Antal et al [27] investigated the effect of $HgCl_2$ and $MeHg^+$ on photosynthetic activity of diatom *Thalassiosira weissflogii* and found that $MeHg^+$ applied in the concentration range 10^{-6}-10^{-7} mol dm^{-3} decreased the photochemical activity of the PS 2 reaction centres in cells of this microalgae after a prolonged lag phase, whereas $HgCl_2$ decreased the activity of PS 2 only at higher concentrations. Similar results were obtained by Graevskaya et al [28]. However, these authors observed that PS 2 inactivation by $MeHg^+$ was not complete and about 10% of the cells kept high level of PS 2 activity, suggesting the adaptation of algae to the $MeHg^+$ treatment. Methylmercury chloride and $HgCl_2$ decreased the rate of PS 2 reparation and increased a heat pathway of excitation dissipation in PS 2 antennae complex. Different toxic effects of mercuric chloride and methylmercuric chloride on the freshwater alga *Poterioochromonas malhamensis* were reported by Röder [29].

The goal of this work is to determine the site and mode of action of organomercury compounds (methylmercuric chloride, phenylmercuric borate, phenylmercuric acetate, phenylmercuric citrate and diphenylmercury) in photosynthetic apparatus of spinach chloroplasts.

Materials and methods

Chemicals and instruments: $HgCl_2$ and the studied organomercury compounds (methylmercuric chloride, phenylmercuric borate, phenylmercuric acetate, phenylmercuric citrate and diphenylmercury) and ascorbic acid were purchased from Centralchem (Slovak Republic). $MgCl_2$, NaCl, saccharose, 2,6-dichlorophenolindophenol sodium salt (DCPIP), 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and dimethyl sulfoxide (DMSO) were purchased from Slavus (Slovak Republic). The spectra of electron paramagnetic resonance (EPR) were recorded by an ERS 230 instrument (ZWG, Akademie der Wissenschaften, Berlin, Germany), which operates in X-range (~ 9.3 GHz). Absorption spectra were recorded by UV-VIS spectrophotometer Genesis 6 Thermo-Scientific, USA.

Chloroplast preparation: Chloroplasts were prepared from spinach (*Spinacia oleracea* L.) purchased locally by the procedure of Walker [30] partly modified by Sersen et al [31] using a TRIS buffer (20 mmol dm^{-3}; pH = 7.0) containing 0.4 mmol dm^{-3} saccharose and 20 mmol dm^{-3} $MgCl_2$. The chlorophyll content was determined according to Wellburn [32].

Measurements of PET: The photosynthetic electron transport through PS 2 and PS 1 in the suspension of spinach chloroplast (30 mg Chl dm^{-3}) was measured in a phosphate buffer (pH = 7.2) containing 5 mmol dm^{-3} $MgCl_2$, 15 mmol dm^{-3} NaCl and 0.4 mmol dm^{-3} saccharose according to the work of Xiao et al [33]. By monitoring the rates of photoreduction of artificial electron acceptor DCPIP (40 μmol dm^{-3}) the electron transport through PS 2 was determined. On the other hand, the PET through PS 1 was monitored by photooxidation of $DCPIPH_2$ in the same buffer as in previous experiment. In the control sample the supply of electrons from PS 2 to PS 1 was stopped by DCMU (20 μmol dm^{-3}) and sodium ascorbate (40 μmol dm^{-3}) as an artificial electron donor of PS 1 and methylviologen (0.1 mmol dm^{-3}) as a final electron acceptor of PS 1 was used in this experiment. The investigated photoprocesses were recorded spectrophotometrically as changes in the absorbance of DCPIP at 595 nm, *ie* as its decrease (in the case of PET through PS 2), or its increase (in the case of PET through PS 1). The incubation time after adding of studied compounds to chloroplast suspension was approximately 1 min in both experiments. The irradiation of the chloroplast suspension was carried out by a 250 W halogen lamp through a 5 cm water filter. The intensity of irradiation was 900 μE m^{-2} s^{-1} PAR (photosynthetically active radiation). Due to low aqueous solubility of studied organomercury compounds, these were dissolved in DMSO. The DMSO content up to 4% did not affect the photochemical activity in spinach chloroplasts.

EPR measurements: The first derivative EPR spectra were recorded at following instrument parameters: microwave power 5 mW; modulation amplitude 0.5 mT; sweep of magnetic field 20 mT; registration time 6 min; recorder time constant 0.5 s. The chloroplast suspensions placed in a flat cell were irradiated directly in the resonator from 0.5 m distance using a 250 W halogen lamp (280 μE s^{-1} m^{-2} PAR) through a 10 cm water filter to exclude warming of the samples. The content of Chl in the samples was 4 g dm^{-3}, the molar ratio Chl : inhibitor was ~ 0.1. The studied organomercury compounds were added into the chloroplast suspension in the form of DMSO solution and the incubation time after adding of studied compounds was approximately 5 min. The DMSO content in the samples (10%) had no observable effect on the EPR spectra of chloroplasts.

All the above mentioned experiments were carried out at a room temperature of 25ºC.

Results

The inhibitory effect of the studied compounds on DCPIP photoreduction**,** which is directly proportional to the oxygen evolution rate, was expressed by IC_{50} value (concentration causing 50% PET inhibition with respect to the untreated control samples). The IC_{50} values of the studied compounds determined for the inhibition of DCPIP photoreduction are presented in Table 1.

Table 1
The inhibition of DCPIP photoreduction by the studied compounds in spinach chloroplasts expressed by IC_{50} values. All compounds were applied in DMSO solution

Compound	IC_{50} [μmol dm^{-3}]
phenylmercuric borate	468
phenylmercuric acetate	657
phenylmercuric citrate	942
methylmercuric chloride	627
diphenylmercury	does not affect
mercuric chloride	58

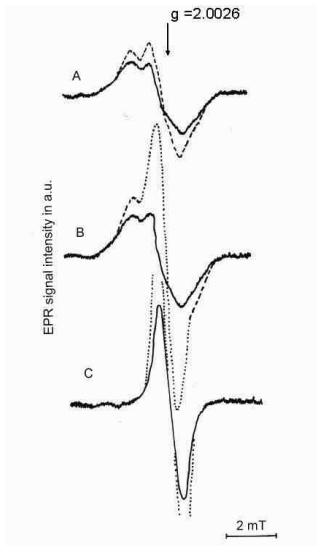

Fig. 1. EPR spectra of untreated chloroplasts (A) and those treated with 0.05 mol dm^{-3} phenylmercuric borate (B) as well as with 0.05 mol dm^{-3} $HgCl_2$ (C). Mercury compounds were applied in DMSO solution. The full line spectra were registered in the dark, the broken ones were registered in the light

EPR spectroscopy was used to find the sites of inhibitory action of the studied compounds in spinach chloroplasts. The EPR spectra of intact chloroplasts are located in the region of free radicals ($g \sim 2$). They consist from two components, so-called signal I and

signal II, belonging to photosystem 1 and 2, respectively [34]. Signal II consists of two parts, namely of EPR signal II_{slow} and EPR signal $II_{very\ fast}$. EPR signal II_{slow} (g = 2.0046, ΔB_{pp} = 1.9 mT) [35] is observable in the dark (Fig. 1A, full line). Signal II_{slow} belongs to the intermediate $D^{\bullet}$ that is a part of PET-chain on the donor side of PS 2. Later, it was found that intermediate $D^{\bullet}$ is the tyrosine radical at the 161st position of D_2 protein [36]. EPR signal $II_{very\ fast}$ (g = 2.0046, ΔB_{pp} = 1.9 mT) [37] which is observable as an increase of signal II in the light is shown in Figure 1A as a difference between the dashed and full lines (*ie* as the difference between curves recorded in the light and in the dark). This signal belongs to the intermediate $Z^{\bullet}$, *ie* to the tyrosine radical at the 161st position of D_1 protein [38] which is also situated on the donor side of PS 2. In Figure 1B,C EPR spectra of spinach chloroplasts treated with 0.05 mol dm^{-3} of phenylmercuric borate and 0.05 mol dm^{-3} $HgCl_2$ are presented. The rest organomercury compounds exhibited similar EPR spectra as are presented in Figure 1B.

Discussion

All studied organomercury compounds, except diphenylmercury, inhibited the rate of PET in spinach chloroplasts. The IC_{50} values determined for the inhibition of DCPIP photoreduction were in a range from 468 to 942 μmol dm^{-3} (Table 1). However, the IC_{50} value determined for phenylmercuric acetate (657 μmol dm^{-3}) was higher than the value found by Honeycutt and Krogmann [21], *ie* 150 μmol dm^{-3}. This discrepancy could be caused by the use of different inhibitor incubation periods: $t \sim 1$ min in our experiment and t = 30 min in the experiments of the above mentioned researchers. The effect of diphenylmercury on DCPIP photoreduction was not measurable due to its very low solubility in the aqueous chloroplast suspension. It was found that in chloroplasts treated with organomercury compounds (500 μmol dm^{-3}) the photooxidation of $DCPIPH_2$ was inhibited only in a range of 10-20%, indicating that only partial PET inhibition through PS 1 occurred. This finding is in accordance with the results of Honeycutt and Krogmann [21].

From Figure 1B it is evident that the studied organomercury compounds did not cause any changes in both components of signal II, *ie* it is possible to suggest, that they did not interact with the intermediates $Z^{\bullet}/D^{\bullet}$. However, some interaction between the photosynthetic apparatus and the studied organomercury compounds occurred, as it is documented in Figure 1B (broken lines). The great increase of signal I in the light can be observed in EPR spectra of chloroplasts treated by organomercury compounds (Fig. 1B, dotted line). Signal I (g = 2.0026, ΔB_{pp} = 0.9 mT) is well observable as the dotted part of the EPR spectra imagined by broken lines in Figure 1B,C. Signal I belongs to the oxidized reaction centre of PS 1, *ie* $P700^{+}$, which is constituted by chlorophyll *a* dimer [34]. The observed increase of the signal I intensity is caused by interruption of the electron transport through the photosynthetic apparatus. Consequently, the reduction of the $P700^{+}$ that was oxidized in the light cannot occur. This effect can be caused by the interaction of organomercury compounds with Q_A or Q_B on the acceptor side of PS 2 as was proposed in the work of Prokowski [19].

According to the findings of Honeycutt and Krogmann [21], ferredoxin on the acceptor side of PS 1 could also be suggested as a probable site of action of organomercury compounds. For verification of this idea we carried out an EPR experiment for monitoring PET through PS 1. The dynamics of the reduction of $P700^{+}$ was investigated by the decay

of the light-induced EPR signal I in the dark, similarly as was carried out in our previous work [17]. Figure 2 presents time dependencies of the decrease of EPR signal I intensity in the dark after switching off the illumination in control and organomercurial-treated chloroplast samples as well. For good registration of signal I in control chloroplasts, these were treated with 0.05 mol dm^{-3} DCMU, a PET inhibitor which interrupts electron transport from PS 2 to PS 1 but does not damage PS 1 [39]. From the results of this experiment it is evident that the exponential decay constants are higher in chloroplasts treated with organomercury compounds than in control chloroplasts. From literature data, it is known that the time dependence of a light-induced signal I after light switch off exhibits bi-phase exponential decay [40]. The fast decay ($\tau_1 \sim 200$ μs) corresponds to the back return of electrons from the ferredoxin terminal acceptor to the PS 1 core $P700^+$ [40]. It could be assumed that the slow phase of signal I intensity decay with $\tau_2 > 1$ s corresponds to $P700^+$ reduction by cyclic electron flow through PS 1, because the non-cyclic electron flow through PS 2 is damaged by DCMU or by studied organomercury compounds. The time constants τ_1 and τ_2 of exponential decay were calculated by the fitting of experimental curves. It was found that the time constants τ_1 for organomercurial-treated chloroplasts were the same as for DCMU-treated chloroplasts. However, the values of τ_2 in chloroplasts treated by organomercury compounds were higher than in chloroplasts with undamaged PS 1. The τ_2 constants were as follows: 1.86 s for DCMU, 2.02 s for phenyl mercury acetate, 2.0 s for phenyl mercuric chloride and 2.31 s for methyl mercuric borate. These results represent very small changes in τ_2 values. On the basis of changes of time constants τ_2, as well as with regard to the fact that $DCPIPH_2$ photooxidation by PS 1 showed only very small decrease by 10-20%, it can be assumed that the studied organomercury compounds damaged PET through PS 1 only weakly. The terminal electron acceptor, *ie* ferredoxin, on the acceptor side of PS 1 can be denoted as the site of action of organomercury compounds. However, we suppose that their main site of action is Q_A or Q_B acceptor on the reducing side of PS 2.

From the above-presented results it follows that the organomercury compounds under study had much smaller inhibitory effect on PET through PS 2 in spinach chloroplasts than $HgCl_2$ with $IC_{50} = 31$ μmol dm^{-3} [16]. The higher inhibitory efficiency of $HgCl_2$ can be connected with the different sites of action of organomercury compounds and $HgCl_2$, respectively. Whereas $HgCl_2$ interacts with the intermediates $Z^{\bullet}/D^{\bullet}$ and with the manganese cluster in the oxygen evolving complex, as was documented by registration of Mn^{2+} ions which were released into the interior of thylakoid membranes [16], the site of the inhibitory action of organomercuric compounds did not occur on the donor side of PS 2 and PET inhibition is probably a result of the interaction of these compounds with Q_A or Q_B in PS 2 and with ferredoxin in PS 1. Because in our previous work chloroplasts were treated with an aqueous $HgCl_2$ solution [16] and in the current experiments they were treated with organomercury compounds dissolved in DMSO, a further experiment was performed with chloroplasts treated with $HgCl_2$ dissolved in DMSO. The corresponding EPR spectrum of chloroplasts treated in this manner is presented in Figure 1C. From this spectrum it is evident that $HgCl_2$ applied without as well as with DMSO exhibits the same effects on chloroplasts, *ie* it interacts with the intermediates $Z^{\bullet}/D^{\bullet}$ and oxidizes PS 1, even in the dark (Fig. 1C full line). However, in the presence of DMSO, a release of Mn^{2+} ions from the oxygen evolving complex was not estimated. Moreover, a decrease of the rate of PET

through PS 2 was observed in chloroplasts treated with $HgCl_2$ dissolved in DMSO (IC_{50} = 58 μmol dm^{-3}; Table 1) in comparison with that obtained without DMSO (IC_{50} = 31 μmol dm^{-3}) [16]. The lower efficacy of $HgCl_2$ applied with DMSO can be caused by a complex formation of $HgCl_2$ with DMSO, in which DMSO acts as an *O*-donor ligand [41].

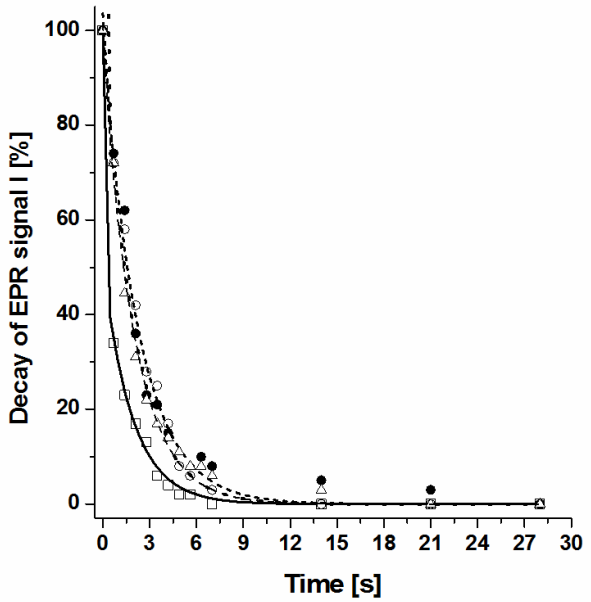

Fig. 2. Decay of EPR signal I intensity of chloroplasts treated with 0.05 mol dm^{-3} of DCMU (open squares, full line), methylmercuric chloride (open circles, dotted line), phenylmercuric borate (dark circles, short dashed line) and phenylmercuric acetate (triangles, dashed line) after switch off light. Time constant of the EPR recorder was zero in these experiments

The lower inhibitory effect of organomercury compounds on PET compared with $HgCl_2$ can be probably caused by the fact that the chemical affinity of organomercury for ligands, including amino acid residues in peptides, is similar to that of Hg^{2+} but the stability constants of methylmercury complexes with these ligands are consistently lower than those of the corresponding Hg^{2+} complexes [42]. This was confirmed by Stary and Kratzer [43] who found that mercury forms thiolate complexes exhibiting extraordinarily high formation constants. Whereas for $HgCys_2$ complexes this value was reported to be about 10^{42}, similar formation constants for methylmercury and phenylmercury achieve only 10^{15} and 10^{16}, respectively.

It was also found that organomercury compounds exhibit higher inhibitory effect on growth of algae [25-28] than on PET in spinach chloroplasts. This difference can be associated with the inhibition of the enzymatic processes connected with algal growth and biosythesis of chlorophyll. Moreover, it is known that the toxicological effect of mercury is intensified due to transformation of organic and inorganic compounds into a more toxic form by the microorganisms [44].

Conclusions

Organomercury compounds phenylmercuric borate, phenylmercuric acetate, phenylmercuric citrate and methylmercuric chloride inhibited PET in spinach chloroplasts. Their IC_{50} values were approximately 10-times higher than the IC_{50} value determined for $HgCl_2$. The lower inhibitory effectiveness of organomercury compounds can be caused by their complexation with amino acid residues in peptides, but their stability constants are consistently lower than those of the corresponding complexes formed with $HgCl_2$. On the basis of the EPR study, we suggest that the sites of action of studied organomercury compounds are Q_A or Q_B quinones on the acceptor side of PS 2 and terminal ferredoxin acceptor in PS 1.

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WPŁYW NIEKTÓRYCH ZWIĄZKÓW RTĘCI NA FOTOSYNTEZĘ W CHLOROPLASTACH SZPINAKU

Abstrakt: Zbadano wpływ pięciu związków rtęcioorganicznych (chlorku metylortęci, octanu fenylortęci, boranu fenylortęci, cytrynianu fenylortęci i difenylortęci) na fotosyntetyczny transport elektronów (PET) w chloroplastach szpinaku. Wartości IC_{50} dla związków rtęcioorganicznych związanych z inhibicją PET w chloroplastach szpinaku zmieniała się w zakresie od 468 do 942 µmol dm^{-3} i była w przybliżeniu o rząd większa od odpowiedniej wartości określonej dla $HgCl_2$, stosowanego również w roztworze DMSO (IC_{50} = 58 µmol dm^{-3}). Ze względu na bardzo małą rozpuszczalność difenylortęci w wodzie odpowiednia wartość IC_{50} nie może być określona. Wyniki badań za pomocą spektroskopii EPR pozwoliły na zaproponowanie prawdopodobnych miejsc działania związków rtęci w procesie fotosyntezy ferredoksyny po stronie akceptora PS 1 i chinonowego akceptora elektronów Q_A lub Q_B po stronie redukującej PS 2.

Słowa kluczowe: spektroskopia EPR, organiczne związki rtęci, fotosyntetyczny transport elektronów