# Concentration-dependent Inhibitory and Stimulating Effects of Amphiphilic Ammonium Salts upon Photosynthetic Activity of Spinach Chloroplasts

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**Abstract.** The effects of piperidinopropylesters of 2-, 3- and 4-alkoxy substituted phenylcarbamic acids (PPACs) on oxygen evolution rate (OER) in spinach chloroplasts were investigated. PPACs show concentration-dependent effects, namely OER inhibition at higher effector concentrations and OER stimulation at lower concentrations, i.e. below the inhibitory concentration range. The inhibitory efficiency of PPACs showed dependence on the alkyl chain length of the alkoxy substituent as well as on the position of this substituent on the benzene ring. Using EPR spectroscopy and fluorescence measurements it was confirmed that the site of PPAC inhibitory action is the donor side of photosystem 2, where $D_1$ and $D_2$ proteins are situated, namely the intermediates $Z^+/Y^+$, and the manganese cluster containing protein as well. The stimulating effects of PPACs on OER in spinach chloroplasts at relatively low effector concentrations are caused by photophosphorylation uncoupling due to protonophore properties of the effectors.

**Key words:** Spinach chloroplasts — Local anaesthetics — Photosynthesis inhibition — Photosynthesis stimulation

## Introduction

Many esters of alkoxy substituted phenylcarbamic acids are membrane-active compounds showing a variety of biological, e.g. local anaesthetic (Gregáň et al. 1993a; Čižmárik et al. 1976; 1992), plant growth regulating (Mitterhauszerová et al. 1991), antimicrobial (Mlynarčík and Čižmárik 1976) and antiarrhythmic (Kozlovský et al. 1982) activities. Their toxicity against *Chlamydomonas reinhardtii* has also been confirmed (Miadoková et al. 1995). These compounds are known to inhibit photosynthetic processes in photosynthesizing organisms as well (Kráľová et al. 1991; 1992a; 1994a,b; Gregáň et al. 1993b). Using EPR spectroscopy it was confirmed that these amphiphilic effectors cause destruction of photosystem (PS) 2 with sub-

sequent release of manganese ions into the interior of thylakoid membranes (Šeršeň et al. 1990; Kráľová et al. 1992a,b). The photosynthesis inhibiting activity of alkoxy substituted phenylcarbamates showed good correlation with the lipophilicity of the respective effector, however the inhibitory activity of 2-substituted derivatives was lower than that of 3- and 4- substituted ones (Kráľová et al. 1992a,b,c; 1994a,b).

Stimulation of photochemical activity of plant chloroplasts in the presence of relatively low concentrations of some amphiphilic compounds has been described repeatedly. As model compounds causing enhancement of oxygen evolution rate in plant chloroplasts, ionic surfactants (Apostolova 1988; Šeršeň and Devínsky 1994; Šeršeň and Lacko 1995) or selected local anaesthetics, e.g. dibucaine or tetracaine (Semin et al. 1989) have been investigated.

Piperidinopropyl esters of 2-, 3- and 4-alkoxy substituted phenylcarbamic acids (alkoxy = methoxy – *n*-decyloxy) (PPACs) have been shown to exhibit pronounced algicidal activity (Kráľová et al. 1995). The present paper is aimed at investigating the concentration-dependent effect of PPACs on oxygen evolution rate in spinach chloroplasts, and to determine the site and the mode of action of these effectors.

## Materials and Methods

The studied piperidinopropyl esters of 2-, 3- and 4-alkoxy substituted phenylcarbamic acids (alkoxy = methoxy – *n*-decyloxy) were prepared according to the method described by Čižmárik et al. (1976). The effects of the compounds tested on the oxygen evolution rate (OER) in spinach chloroplasts prepared according to Šeršeň et al. (1990) were investigated spectrophotometrically (Specord UV-VIS, Zeiss Jena, Germany) in the presence of the artificial electron acceptor 2,6-dichlorophenol-indophenol (DPIP) using the method described by Kráľová et al. (1992a). The inhibitory activity of the studied effectors was expressed in terms of $pI_{50}$ values, i.e. as the negative logarithm of the molar PPAC concentration inducing a 50% decrease of OER with respect to the respective control sample. These measurements were performed at room temperature using chloroplast suspensions containing 20 mg chlorophyll (Chl) per liter. The reproducibility of repeated measurements was in the range of ±10%.

Fluorescence measurements were performed with an F-2000 spectrophotometer (Hitachi, Japan) at room temperature. Chloroplast suspensions containing 10 mg Chl per liter were excited at 436 nm, i.e. at the wavelength causing mainly excitation of $Chl_a$, using 10 nm slit, and changes in the intensity of Chl emission in the 600–800 nm range produced by the presence of the effector were recorded. Prior to the measurements the samples were kept in the dark for 10 min.

EPR measurements were carried out with an ERS 230 (WG, Akademie der Wissenschaften, Berlin, Germany), operating in X-band at 5 mW microwave power and 0.5 mT modulation amplitude. EPR spectra of untreated spinach chloroplasts

and those obtained in the presence of the studied compounds (0.05 mol/l) were recorded in the dark and in the light at room temperature. Chl content in the samples was 4 g/l. The samples were irradiated with a 250 W halogen lamp through a water filter.

## Results

The effects of PPACs on OER in spinach chloroplasts are documented in Fig. 1. From this Figure it is obvious that the investigated effect of the studied effectors is concentration-dependent; i.e. it is inhibitory at relatively high effector concentrations, whereas stimulating effects can be observed at lower concentrations. It is also shown that no additional OER increase due to PPACs action can be obtained in chloroplast suspensions in which all sites of photophosphorylation were uncoupled by 5.5 mmol/l $NH_4Cl$.

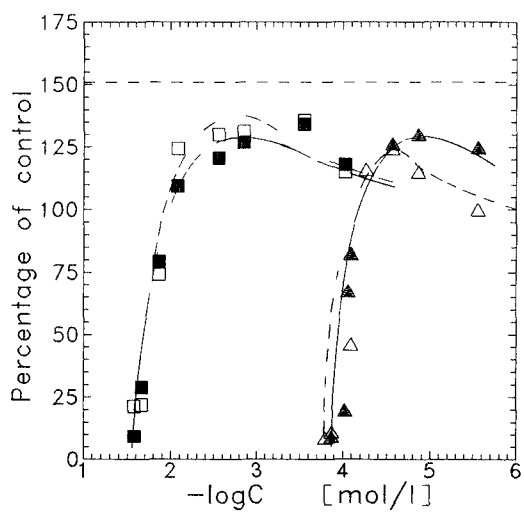


**Figure 1.** The dependence of the DPIP photoreduction rate upon PPAC concentration in a suspension of spinach chloroplasts expressed as the percentage of the untreated control without (empty symbols, dashed lines) and in the presence of 5.5 mmol/l $NH_4Cl$ (full symbols, full lines) 3-ethoxy- (squares) and 3-nonyloxy- derivatives (triangles). The horizontal dashed line at 154% corresponds to OER in chloroplasts pre-uncoupled by 5.5 mmol/l $NH_4Cl$.

The fluorescence study showed that in the presence of PPACs used at inhibitory concentrations the intensity of the Chl emission band at 686 nm exhibits a time-dependent decrease. Fig. 2 illustrates the effect of a model effector, 3-nonyloxy PPAC derivative, all the other compounds studied exhibited similar behavior. In the presence of OER-stimulating PPAC concentrations, the changes in fluorescence of the chloroplast suspension were very small (ca 2%) compared to the control sample.

All components of signal II in the EPR spectra of spinach chloroplasts disappeared in the presence of PPAC effectors (Fig. 3*B*, full line). On the other hand, the

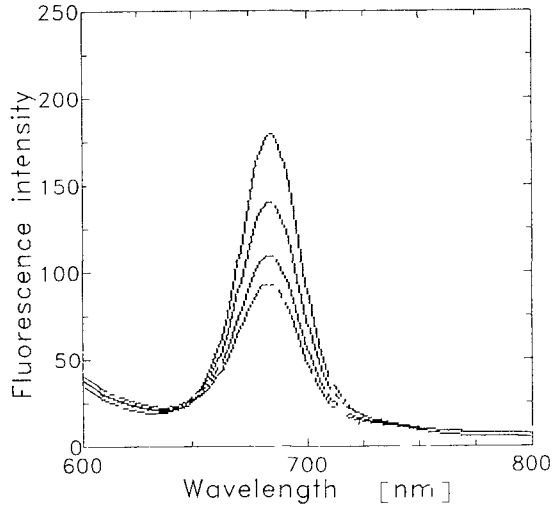


**Figure 2.** The fluorescence spectra of untreated suspension of spinach chloroplasts and in the presence of 30 $\mu$mol/l of 3-nonyloxy PPAC derivative 10, 20, and 30 min after addition of the effector ($\lambda_{ex}$ = 436 nm) (from top to bottom).

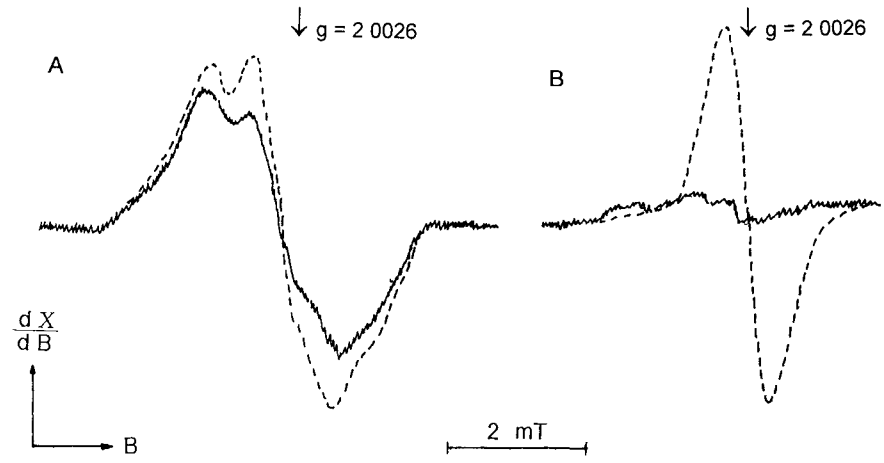


**Figure 3.** EPR spectra of untreated spinach chloroplasts ($A$) and of chloroplasts treated with 0.05 mol/l 2-nonyloxy PPAC derivative ($B$). The spectra were recorded in the dark (full lines) and in the light (dashed lines). The dashed line in B was recorded at 0.5 amplification.

intensity of signal I in the light was very high (Fig. 3$B$, dashed line). These EPR spectra are of the same shape and nature as those observed with chloroplast suspensions containing piperidinoethylesters, N-ethyl-2-pyrrolidinylmethylesters and N-alkyl-4-piperidylesters of alkoxy substituted phenylcarbamic acids (for detailed

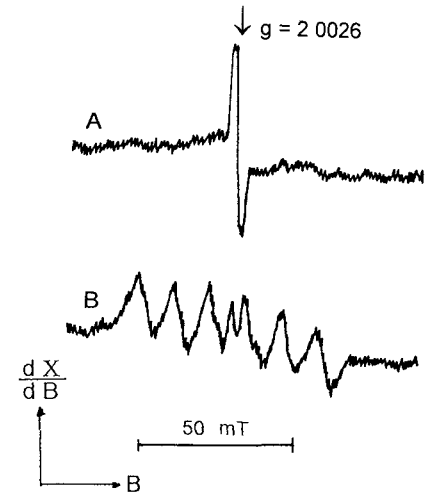


**Figure 4.** EPR spectra of $Mn^{2+}$ ions in untreated chloroplasts (line *A*) and in chloroplasts treated with 0 05 mol/l 2-nonyloxy PPAC derivative (line *B*)

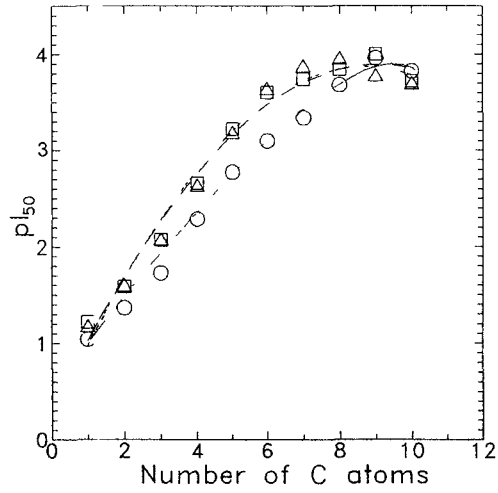


**Figure 5.** The dependence of OER in spinach chloroplasts (expressed in terms of $pI_{50}$ values) upon the alkyl chain length of PPAC alkoxy substituent (2-(circles, full line); 3-(squares, broken line) and 4-substituted (triangles, dashed line) derivatives).

description see Kráľová et al. 1992a,b). Similarly to the above mentioned phenylcarbamates PPAC cause release of manganese ions into the thylakoid membranes as well. This is manifested in EPR spectra of $Mn^{2+}$ as six lines of fine structure (Fig. 4).

The dependence of $pI_{50}$ values for PPACs on the alkyl chain length of the PPAC alkoxy substituents is shown in Fig. 5. The inhibitory activity of the studied effectors increases with the increasing length of the PPAC alkoxy substituent

up to heptyloxy – octyloxy derivatives; however, further increasing the effector lipophilicity results in a decrease of activity. Also, it was confirmed that the inhibitory activity of 2-substituted PPAC derivatives is lower than that of 3- and 4-substituted ones (Fig. 5).

## Discussion

By studying the effect of PPAC on OER in a wide concentration range of the effector it was found that at lower than inhibitory effector concentrations, PPACs showed stimulating effect on OER in spinach chloroplasts (Fig. 1). This stimulation of OER can be a result of two mechanisms. One of them is uncoupling of photophosphorylations due to protonophore properties of PPAC. Thus, PPACs promote passive $H^+$ diffusion with subsequent dissipation of electrochemical proton gradient between the inside and the outside of the thylakoid membranes resulting in faster course of the photosynthetic electron transport (Semin et al. 1989; Šeršeň and Kráľová 1994). On the other hand, the enhancement of OER by PPAC could be associated with PPAC membrane-active properties: at certain effector concentrations below the inhibitory ones changes in the arrangement of the thylakoid membranes may take place which result in an increase of the photosynthetic electron transport (Apostolova 1988; Šeršeň and Devínsky 1994; Šeršeň and Lacko 1995).

To determine which of the above-mentioned mechanisms is responsible for the enhancement of OER a further experiment was done. Using $NH_4Cl$, a well-known uncoupler of photophosphorylation (Buschmann and Grumbach 1985), OER in chloroplast suspension was stimulated to the maximum value and subsequently PPAC was added. The results of this experiment are illustrated in Fig. 1. If OER stimulation by PPACs were also associated with a change in the arrangement of the thylakoid membranes, a further increase of OER would be expected after addition of a certain amount of PPAC to chloroplasts in which all sites of photophosphorylation were uncoupled by $NH_4Cl$. However, from Fig. 1 it is evident that independently of the length of their alkoxy substituent the studied derivatives did not cause any additional enhancement of OER; i.e. it is the uncoupling mechanism that is responsible for OER stimulation by PPACs.

The effect of PPAC on the photosynthetic centres of chloroplasts was investigated by studying $Chl_a$ fluorescence. The intensity of the emission band at 686 nm, belonging to the pigment-protein complex in PS 2 (Atal et al. 1991) showed a time-dependent decrease (Fig. 2) suggesting PS 2 as the site of action of the studied effectors.

In the presence of PPACs the EPR signal belonging to the intermediate $Z^+/Y^+$ disappeared (Fig. 3*B*, full line) and a new EPR signal belonging to free $Mn^{2+}$ ions was observed (Fig. 4*B*). It is known that the intermediate $Z^+$ belongs to tyrosine

161 and the $Y^+$ to tyrosine 160 which are located in the $D_1$ and $D_2$ polypeptides situated on the donor side of PS 2 (Barry and Babcock 1987; 1988; Noren et al. 1991; Noren and Barry 1992). From the above mentioned findings and our results it can be concluded that the inhibitory effect of PPACs at higher effector concentrations is due to a damage of the proteins located on the donor side of PS 2, namely $D_1$ and $D_2$ and the protein containing manganese cluster which is also located on the donor side of PS 2 (Govindjee and Wasielewski 1989). The intensity of the EPR signal I belonging to PS 1 of chloroplasts treated with inhibiting amounts of PPAC became very high in the light (Fig. 3*B*, dashed line). This means that oxidized PS 1 cannot be reduced while the PPAC-damaged PS 2 is unable to supply electrons to PS 1. Consequently, the PPAC site of action is only limited to PS 2, and these effectors do not affect PS 1.

Using sym-diphenylcarbazide (DPC), an artificial electron donor for P680 with the site of action known to be the intermediates $Z^+/Y^+$ (Jagerschöld and Styring 1991) it can be determined whether PPAC effectors damage the core of PS 2. The addition of 0.2 mmol/l DPC to PPAC-inhibited chloroplasts (approximately 90% inhibition as compared to the control) caused complete restoration of the photosynthetic electron transport through P680 to DPIP. From these results it can be assumed that PPACs do not interfere with the core of PS 2.

The relationship between the alkyl chain length of PPACs and their effect on OER inhibition in spinach chloroplasts exhibits a quasi-parabolic course (Fig. 5). It is known that the longer the alkyl chain the higher the lipophilicity of the effector molecule and therefore its affinity to the lipidic part of biological membranes. The results discussed above showed that the decrease of OER is due to PPAC interaction with proteins which are situated on the donor side of PS 2. Therefore, it is obvious that the intensity of PPAC effect on OER will depend on the competition between PPAC affinity to the lipidic and the protein part of the photosynthetic membrane. The lipophilicity of PPACs with short alkyl chain is low, resulting in their limited transition through the membranes and in their more difficult access to interaction with PS 2-proteins, situated on the inner side of the thylakoid membranes (Ort and Govindjee 1987). On the other hand, due to their higher lipophilicity PPACs with long alkyl chain have a large partition coefficient expressing effector partitioning between the biological membrane and the aqueous phase (Balgavý et al. 1992). As a result, long-chain PPACs predominantly remain incorporated in the lipidic part of the membrane and only a limited number of effector molecules will arrive at the membrane proteins. Consequently, the highest activity will be exhibited by effectors with sufficiently high lipophilicity for securing their passage through the lipidic membranes and simultaneously enabling sufficiently high concentrations of PPAC molecules in the aqueous phase to interact with proteins. Also, local anaesthetic (Čižmárik et al. 1992) and algicidal and antifungal activity of PPAC (Kráľová et al. 1995) showed quasi-parabolic course in dependence on the alkyl chain length

of the alkoxy substituent. These findings together with the above discussed results concerning OER inhibition in spinach chloroplasts by these effectors are in good accordance with the results obtained for structurally similar local anaesthetics (Kráľová et al. 1992a,b, 1994a,b). The lower photosynthesis inhibiting activity of 2-substituted PPAC derivatives compared to that of 3- and 4-substituted ones (Fig. 5) is probably due to secondary sterical effect, namely to a distortion of the benzene ring plane against that of the carbamate group (Čižmárik et al. 1976).

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