Interference by Nickel(II) Salts and Their 5-Methylimidazole-4-carboxylate Coordination Compounds on the Chloroplast Redox Chain

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Coordination Compounds, Photosynthesis, Ni(II), Emizco, Hill Reaction Inhibitors

Nickel(II) salts and their coordination compounds with ethyl 5-methylimidazole-4-carboxylate (emizco), \([\text{Ni}(\text{emizco})_2\text{Cl}_2]\), \([\text{Ni}(\text{emizco})_2\text{Br}_2]\), \([\text{Ni}(\text{emizco})_2(\text{H}_2\text{O})_2](\text{NO}_3)_2\text{H}_2\text{O}\), \([\text{Ni}(\text{NO}_3)_2]\), inhibit photosynthetic electron flow (basal, phosphorylating and uncoupled) and ATP-synthesis, therefore behave as Hill reaction inhibitors. Coordination compounds are more potent inhibitors than the salts. It was found that the target for \(\text{NiCl}_2\); \(\text{NiBr}_2\) and \(\text{Ni}(\text{NO}_3)_2\) is at the b6f level. On the other hand, the complexes \([\text{Ni}(\text{Emizco})_2\text{Cl}_2]\), \([\text{Ni}(\text{Emizco})_2\text{Br}_2]\) and \([\text{Ni}(\text{emizco})_2(\text{H}_2\text{O})_2](\text{NO}_3)_2\text{H}_2\text{O}\) binding sites are located at \(Q_6(D1)\)-protein and b6f level. Therefore, they have a common inhibition site located at b6f avoiding the POH oxidation. The Qb inhibition site was corroborated by variable chlorophyll \(a\) fluorescence yield \((V(j))\). The emizco ligand has no activity on photosynthetic electron flow.

Introduction

Continuing our earlier work related with the study of the effect of transition metals ions coordination compounds on different photosynthetic activities (Barba-Behrens et al., 1993; Fernández-Vargas et al., 1995), we report the behaviour of nickel coordination compounds with the ligand ethyl 4-methyl-imidazolecarboxylate (emizco) on photosynthesis. Their effect was compared with that of the ligand and nickel(II) salts. Recently, we found that quinic acid uncouples photophosphorylation from photosynthetic electron flow, while Co(II) coordination compounds enhance this activity (Barba-Behrens et al., 1991). Emizco was chosen as a simple imidazolic derivative that may act as a chelate towards metal ions, similarly to 2-methyl-5-nitroimidazole (Barba-Behrens et al., 1991). It is known that imidazolic derivatives have biocidal properties, they are extensively used in the pharmaceutical (Bennett, 1990) and agrochemical industries (Garaboyes, 1982; Parsons et al., 1990). Emizco derivatives present antiviral (Alonso et al., 1985) and herbicidal (Beck et al., 1979) activities. Nickel(II) is an essential micronutrient for legumes and suggested possible essentiality for all higher plants (Farago et al., 1988). There is evidence that nickel is required in microbial urea-utilising plants (Farago et al., 1988). It is reported to be mutagenic and carcinogenic causing chromosome aberrations and micronucleus formation (Leonard et al., 1981) and it is toxic at 0.1–
1 mM concentrations, which inhibited seed germination of *Helianthus annuus* (Chakravarty et al., 1992). Photosynthesis and respiration of lichens have been shown to be affected by metals, it was found that nickel(II) is the less toxic one (Farago et al., 1988). In this work we studied the effect of nickel(II) salts, emizco and their coordination compounds at lower concentrations (0–500 μM). Nickel(II) salts and emizco were used as control experiment in order to see if the ligand modified the potency of the salt. As far as we know the effects of these compounds on photosynthesis in chloroplasts in higher plants have not been investigated.

**Materials and Methods**

**Freshly lysed chloroplasts isolation and chlorophyll determination**

Intact chloroplasts were isolated from spinach leaves obtained from local markets as described earlier (Calera et al., 1995; Mill et al., 1980; Saha et al., 1971). Chloroplasts were suspended in the following medium: 400 mM sucrose, 10 mM KCl, 5 mM MgCl₂ and 30 mM tricine buffer (pH 8 with the addition of KOH). They were stored as a concentrated suspension in the dark for 1 hour at 0°C. Intact chloroplasts were efficiently lysed to yield free thylakoids previous to each experiment by incubating them in the following medium: 100 mM sorbitol, 10 mM KCl, 5 mM MgCl₂ and 30 mM tricine buffer (pH 8 with the addition of KOH) for electron transport measurements. For ATP synthesis determination the same medium was used but tricine buffer concentration was 1 mM (Dilley, 1972). Chlorophyll was determined according to the method of Arnon (1949).

**Measurement of electron transport and ATP synthesis**

ATP-synthesis was determined titrimetrically using a microelectrode Orion Mod. 8103 Ross connected to a Corning potentiometer Model 12, with expanded scale as reported by Dilley (Dilley, 1972). The pH changes were registered using a Gilson recorder. The ATP-synthesis reaction medium used contained 100 mM sorbitol, 10 mM KCl, 5 mM MgCl₂, 0.5 mM KCN, 50 μM MV, 1 mM HEPES-KOH (pH 8.0) where the intact chloroplasts were freshly lysed.

Photosynthetic non-cyclic electron transport in the presence of methylviologen was monitored with YSI (Yellow Springs Instrument C) model 5300 oxygen monitor using a Clark electrode in a temperature regulated flask at 20°C. The reaction medium contained 100 mM sorbitol, 10 mM KCl, 5 mM MgCl₂, 50 μM MV, 0.5 mM KCN, 15 mM HEPES-KOH, (pH 8.0), chloroplasts were added to give a Chl concentration of 20 μg/ml. The sample was illuminated for 1 minute in presence or absence of 6 mM NH₄Cl (Calera et al., 1995; Saha et al., 1971).

PSII was measured by photoreduction of DCPIP supported O₂ evolution monitored polarographically. The reaction medium for assaying PSII activity contained the same whole-chain electron transport medium (H₂O→MV) above mentioned without methylviologen, but in the presence of 1 μM DBMIB, 100 μM DCPIP, 300 μM [Fe(CN)₆]₄⁻ and 6 mM NH₄Cl. Uncoupled electron transport from water to DCBQ (Yruela et al., 1991) was measured with a reaction mixture as in photosystem II, with addition of 100 μM DCBQ, 1 μM DBMIB and 6 mM NH₄Cl without DCPIP and [Fe(CN)₆]₄⁻.

Photosystem I electron transport was determined in a similar form to non-cyclic electron transport. The following reagents were added: 100 μM DCPIP, 300 μM ascorbate, 10 μM DCMU and 6 mM NH₄Cl (Allen et al., 1974).

PS I electron transport from PMS/ASC to MV was measured using KCN-poisoned chloroplasts with 500 μM PMSred/1000 μM ascorbate as the electron donor to P700, MV as PS I electron acceptor, as well as 10 μM DCMU, 6 mM NH₄Cl and 1 μM DBMIB to fully inhibit any electron flow prior to PC. Cyanide-treated chloroplasts were prepared by incubating chloroplasts for 30 min at 0°C in a 30 mM KCN and then centrifuged at 8000×g (Sorvall Super T21) for 1 min and resuspended in the reaction medium (Ouitrakul et al., 1973). Moreover, EPR spectroscopy confirmed the ability of reduced PMS to interact directly with P₇₀₀ (Izawa et al., 1973).

**Chl a fluorescence measurements**

Freshly lysed chloroplasts aliquots containing 15 μg of Chl were suspended in electron transport
medium and transferred by gravity onto filter paper
with a dot-blot apparatus (Bio-Rad USA) to ensure an homogeneous and reproducible distribution of thylakoids in the filter paper. Thylakoids blots were transferred immediately to vials containing 3 ml of different solutions of the tested compounds and incubated for 5 min in the dark. Chl a fluorescence induction curves were measured at room temperature with a Plant Efficiency Analyser (PEA)(Hansatech UK), as described (Strasser et al., 1995).

Kinetic analyses of the relative variable fluorescence \([V(t)=(Ft-Fo)/Fm-Fo]\) were performed by deconvolution of normalised induction curves, employing a non-linear fitting procedure.

**Preparation of Ni-emizco compounds**

\[[\text{Ni(emizco)}_2\text{Cl}_2], \ [\text{Ni(emizco)}_2\text{Br}_2] \text{ and } \ [\text{Ni(emizco)}_2\text{(H}_2\text{O})_2](\text{NO}_3)_2\cdot\text{H}_2\text{O} \]

were prepared as described below.

The nickel(II) coordination compounds were prepared using methanol as solvent and 1:2 ligand:Ni molar ratio was employed (mmol scale). The metal salts, NiCl\(_2\cdot6\text{H}_2\text{O}, \ \text{NiBr}_2\cdot3\text{H}_2\text{O},\ \text{Ni(NO}_3)_2\cdot6\text{H}_2\text{O} \) (Baker) were dissolved in 15 cm\(^3\) of hot methanol and added to a solution of emizco (Aldrich) in 15 cm\(^3\) of hot methanol. The reaction mixture was refluxed for ca 5 h and then allowed to stand at room temperature for 3 weeks. The resulting precipitates were filtered, washed and dried. Nickel(II) coordination compounds were characterised by elemental analyses, magnetic susceptibility, IR and UV-Vis spectroscopy.

**Results and Discussion**

Geometry and stability of nickel(II) coordination compounds with emizco

These coordination compounds have octahedral geometry in solid state and in aqueous solution as shown in Fig. 1. The emizco ligand is coordinated to nickel(II) through the imidazolic nitrogen and the oxygen from the ester group, behaving as a chelating ligand, therefore yielding a very stable compound. Consequently, the ligand is not substituted from the complexes; however the initially coordinated chloride and bromide anions were exchanged by water molecules as can be inferred from the UV-visible absorption spectra in solution and in solid state (diffuse reflectance). In the compound \([\text{Ni(emizco)}_2\text{(H}_2\text{O})_2](\text{NO}_3)_2\cdot\text{H}_2\text{O}\) the nitrate groups were not coordinated from the beginning.

It was shown that the buffer did not substitute any ligand from the nickel(II) coordination sphere. Experiments using different buffering sub-

\[[\text{Ni(emizco)}_2\text{(H}_2\text{O})_2](\text{NO}_3)_2\]

![Fig. 1. Structure of octahedral nickel(II) coordination compounds.](image)
stances: HEPES, tricine and Tris at various concentrations were carried out. The concentration of the buffer was varied from 20–40 mM, for two different pH, 7.0 and 8.0. The UV-visible spectra remained unchanged for solutions of increasing buffer concentration. Therefore, it is assumed that in the photosynthetic experiments the nickel(II) complexes are stable in aqueous media at least for two days, as determined from kinetic studies.

**ATP formation and whole chain electron transports**

The degree of inhibition of the entire electron transport chain rate of spinach thylakoids was measured with emizco, nickel(II) salts and their coordination compounds. Emizco lacks any effect on photosynthetic activities. Figure 2A shows the inhibiting effect of increasing concentration of NiCl<sub>2</sub> on methylviologen photoreduction with water as electron donor. Methylviologen photoreduction and its auto-oxidation with oxygen in the medium that results in O<sub>2</sub> uptake by isolated freshly lysed intact chloroplasts, was inhibited. The results obtained indicate that NiCl<sub>2</sub> act as Hill reaction inhibitors, since it inhibited basal, phosphorylating and uncoupled conditions. In order to know if emizco modifies the activity of the nickel(II) ion in the coordination compound, the effect of its complexes on electron flow was tested. Figure 2B, shows the effect of increasing concentration of the [Ni(emizco)<sub>2</sub>Cl<sub>2</sub>] on basal, phosphorylating and uncoupled electron flow. Electron flow under all conditions was partially inhibited, and the extent of inhibition (up to 50%) increased with concentration from 0 to 500 μM. The extent of inhibition by the coordination compounds suggests that these compounds do not interact at the Q<sub>1</sub> site of the D<sub>1</sub> protein interacting with another site of D<sub>1</sub>.

Electron flow is coupled to ATP-synthesis and the energy transduction theory of Mitchell (Mitchell, 1961) has been proposed to account for the mechanism of coupled electron transport and ATP-synthesis, therefore any chemical that inhibits electron flow will inhibit photophosphorylation, as is the case of the nickel(II) salts and their complexes. The ATP-synthesis inhibition order is as follows: 46% and 17% at 500 μM for [Ni(emizco)<sub>2</sub>Cl<sub>2</sub>] and for NiCl<sub>2</sub> respectively. It is possible that coordination compounds are more hydrophobic than the salts therefore allowing them to reach the target more easily. In order to localise the inhibition site of NiCl<sub>2</sub>, NiBr<sub>2</sub>, Ni(NO<sub>3</sub>)<sub>2</sub>, [Ni(emizco)<sub>2</sub>Cl<sub>2</sub>], [Ni(emizco)<sub>2</sub>Br<sub>2</sub>], [Ni(emizco)<sub>2</sub>-

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![Graph](image-url)

**Fig. 2.** Photosynthetic electron transport from water to MV on freshly lysed spinach chloroplasts: basal (■), phosphorylating (●) and uncoupled (▲) electron transport rates. In the presence of (A) NiCl<sub>2</sub> and (B) [Ni(emizco)<sub>2</sub>Cl<sub>2</sub>]. Control average rate values are 311, 640, 1018 μequiv. e<sup>-</sup>/mg chl per h., for basal, phosphorylating and uncoupled electron flows, respectively. Other conditions as described in Material and Methods. Each curve is the average of three replicates.
(H₂O)₂[(NO₃)₂·H₂O and Ni(NO₃)₂ on the electron transport chain, their effect on photosystem I and II were studied.

**PSII-supported electron transport**

[Ni(emizco)Cl₂], partially inhibited uncoupled PSII electron flow from water to DCPIP or DCBQ (Table I). The PSII inhibition was 50% at 500 µm (178 µequiv. e⁻ h⁻¹ mg Chl⁻¹, as compared with 356 µequiv. e⁻ h⁻¹ mg Chl⁻¹ for the control). Noteworthy, nickel(II) salts do not affect this span of the PSII electron transport chain, as the rate of electron transport from H₂O to DCBQ is the same in the presence or absence of the salts, i.e. 356 µequiv. e⁻ h⁻¹ mg Chl⁻¹.

To further localise the inhibition site of nickel complexes on PSII, electron transport from water to SiMo in presence of 10 µm DCMU, was measured. Results show that these complexes did not inhibit this span of the electron transport chain (Table I). It is known that SiMo accepts electrons through QA level, therefore, it is concluded that one of the targets for [Ni(emizco)₂Cl₂], [Ni(emizco)Br₂], [Ni(emizco)₂(H₂O)₂](NO₃)₂·H₂O and Ni(NO₃)₂ is located at the Qₐ-protein or D₁-protein level.

**PSI-supported electron transport**

To determine the target of NiCl₂ and [Ni(emizco)₂Cl₂] beyond Qₐ their effect on uncoupled PSI activity from reduced DCPIP to MV (plus 10 µm DCMU) were determined. PSI electron transport was partially inhibited, i.e. 38% at 500 µm by NiCl₂ suggesting that the inhibition site for these salts is on PSI, as well as in [Ni (emizco)₂Cl₂] (Table I).

Due to the fact that nickel(II) salts do not affect PSII activity and partially inhibit PSI on one hand, on the other, their emizco coordination compounds show only partial inhibition on PSI, their behaviour from PQ pool to F₅ were tested. This was done adding TMQH₂ as electron donor. The results show that this activity was inhibited by the salts and their coordination compounds i.e. 50%, 500 µm which is the same inhibition percentage of electron flow from reduced DCPIP to MV, thus indicating that the interaction target is located at b₆f level.

The span of PSI electron transport from P₇₀₀ to MV (adding PMS⁻ as electron donor) was studied for all compounds. The results show that this span of electron flow was not affected by the coordination compound or nickel(II) salt. Since PSI activity from DCPIPred to MV was inhibited by nickel(II) salts and coordination compounds, suggesting that their target is located at b₆f complex. Therefore, we may conclude that NiCl₂ have only one target. On the other hand, the [Ni(emizco)₂Cl₂] binding sites are located at Qₐ-protein or b₆f level.

**Effect of Ni²⁺ and their coordination compound on Chl fluorescence**

Chl a fluorescence induction curves of thylakoids show the polyphasic sequence of transients (OJIP) described for plants, green algae and cyanobacteria (Iglesias-Prieto, 1995; Govindjee, 1995). This series of transients reflects the sequential reduction of the electron acceptor pool of PSII (Strasser et al., 1995). Addition of 50 µm DCMU results in transformation of the regular OJIP sequence into an OJ sequence. Inhibition of PSII electron transport at the Qₐ-protein site by

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uncoupled</th>
<th>PSII</th>
<th>PSI</th>
<th>TMQH₂ → MV</th>
<th>H₂O → DCBQ</th>
<th>PMS → MV</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1018</td>
<td>0%</td>
<td>467</td>
<td>0%</td>
<td>1800</td>
<td>0%</td>
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<tr>
<td>NiCl₂</td>
<td>407</td>
<td>60%</td>
<td>1116</td>
<td>38%</td>
<td>134</td>
<td>50%</td>
</tr>
<tr>
<td>[Ni(emizco)₂Cl₂]</td>
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<td>85%</td>
<td>234</td>
<td>50%</td>
<td>174</td>
<td>35%</td>
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<tr>
<td>NiBr₂</td>
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<td>60%</td>
<td>1224</td>
<td>32%</td>
<td>134</td>
<td>50%</td>
</tr>
<tr>
<td>[Ni(emizco)₂Br₂]</td>
<td>163</td>
<td>84%</td>
<td>248</td>
<td>47%</td>
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<td>38%</td>
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<tr>
<td>Ni(NO₃)₂</td>
<td>407</td>
<td>60%</td>
<td>1350</td>
<td>25%</td>
<td>134</td>
<td>50%</td>
</tr>
<tr>
<td><a href="NO%E2%82%83">Ni(emizco)₂(H₂O)₂</a>₂·H₂O</td>
<td>204</td>
<td>80%</td>
<td>272</td>
<td>42%</td>
<td>187</td>
<td>30%</td>
</tr>
<tr>
<td>Emizco</td>
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<td>0%</td>
<td>467</td>
<td>0%</td>
<td>1800</td>
<td>0%</td>
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</table>

Table I. Treatment of nickel salts and their coordination compounds at 500 µm on electron transport rate uncoupled, PSII, PSI and partial reactions. Left in µequiv. e⁻/mg Chl per h. Right in per cent of inhibition.
DCM U results in the rapid accumulation of QA\textsuperscript{−} during the first 2 ms of the induction curve. In contrast, control thylakoids require approximately 900 ms to completely close all PSII reaction centres. Increases in the relative variable fluorescence yield at the transient $J[V(J)]$ as a function of $[\text{Ni(emizco)}_2\text{Cl}_2]$ concentration are indicative of a loss in $Q_A\text{−}$ re-oxidation capacity similar to that observed in DCMU-treated thylakoids (Fig. 3). Consistent with the electron transport determinations, thylakoids exposed to different concentrations of $[\text{Ni(emizco)}_2\text{Cl}_2]$ showed a significant concentration-dependent reduction in their relative $Q_A\text{−}$ re-oxidation capacity (Fig. 4). Polarographic determinations of the inhibition of PSII electron transport activity from water to DCBQ as a function of $[\text{Ni(emizco)}_2\text{Cl}_2]$ (Table I), coordination compound concentration (up to 500 $\mu$M) is correlative with the accumulation of $Q_A\text{−}$ shown by the Chl a fluorescence analyses (Fig. 4). On the other hand, addition of various concentrations of metal salts did not result in any detectable variations in the fluorescence characteristics of the thylakoids. These observations strongly suggest that the target site of the coordination compound is located at the acceptor side of PSII at the $Q_B$-protein.

Further support for this interpretation was obtained by the kinetic analyses of Chl a fluorescence induction curves. The rise of $V$ during the first 2 ms of the induction curve requires two components to be accurately described (Fig. 5A and 5B). An exponential component with rate constants between 0.500 and 0.730 ms\textsuperscript{−}1 and a sigmoidal one with time constants close to 0.09 ms\textsuperscript{−}1 (Table II). Kinetic analyses indicate that the increase in the fluorescence yield during the $J$ transient after DCMU infiltration, results from increments in the relative amplitude of both components with major changes in their rate constants (Table II).

Analogous responses were obtained for $[\text{Ni(emizco)}_2\text{Cl}_2]$, $[\text{Ni(emizco)}_2\text{Br}_2]$ and $[\text{Ni(emizco)}_2\text{(H}_2\text{O})_2](\text{NO}_3)_2\cdot\text{H}_2\text{O}$ infiltrated thylakoids. Although small variations in the rate constants were also detected, the main characteristics of the fluorescence rise during the $J$ event are the dif-
Fig. 5. Deconvolution of the photochemical phase of the induction curve of spinach thylakoids showing the two components (one sigmoidal and one exponential, solid line) needed to describe their fluorescence amplitude rise. Crosses For simplicity only one third of the data are presented. (A) Control thylakoids. (B) Thylakoids infiltrated with 1500 μM [Ni(Emizco)2Cl2].

Table II. Kinetic analyses of the relative variable fluorescence (V) yields during the first 2 ms of the induction curve. Values are means of 4 replicates. Values were obtained by numeric deconvolution. Residuals vary from -0.002 to 0.39.

<table>
<thead>
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<th>Sigmoidal Component</th>
<th>Exponential Component</th>
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<tbody>
<tr>
<td></td>
<td>Amplitude (relative)</td>
<td>Time constant (ms⁻¹)</td>
</tr>
<tr>
<td>[Ni(Emizco)2Cl2]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.201</td>
<td>0.084</td>
</tr>
<tr>
<td>100 μM</td>
<td>0.251</td>
<td>0.084</td>
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<tr>
<td>250 μM</td>
<td>0.276</td>
<td>0.080</td>
</tr>
<tr>
<td>500 μM</td>
<td>0.280</td>
<td>0.088</td>
</tr>
<tr>
<td>DCMU (50 μM)</td>
<td>0.446</td>
<td>0.103</td>
</tr>
<tr>
<td>[Ni(Emizco)2Br2]</td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.206</td>
<td>0.098</td>
</tr>
<tr>
<td>100 μM</td>
<td>0.240</td>
<td>0.096</td>
</tr>
<tr>
<td>250 μM</td>
<td>0.252</td>
<td>0.096</td>
</tr>
<tr>
<td>500 μM</td>
<td>0.255</td>
<td>0.093</td>
</tr>
<tr>
<td>DCMU (50 μM)</td>
<td>0.446</td>
<td>0.099</td>
</tr>
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</table>

The concentration-dependent increases in the amplitude of the sigmoidal components are smaller than those observed for the exponential components. They appear to be saturated at concentrations above 100 μM. Exposure of thylakoids to solutions of the coordination compounds (concentrations higher than 700 μM) resulted in increments in the amplitude of the exponential components. The amplitudes are comparable to those observed for DCMU-infiltrated thylakoids. PSII heterogeneity has been interpreted as the result of the presence of two populations of PSII with different optical cross-section and connectivities (Melis et al., 1983), these two populations also have different distributions in the thylakoid membranes. The differential sensitivity of both types of PSII to Ni(II)-coordination compounds, suggest that the complexes can not infiltrate efficiently the regions of the thylakoids where the PSII with sigmoidal kinetics are located.

Apparantly, neither the anions (Cl-, Br-, NO₃-) nor emizco by themselves are important for the inhibition of electron transport (Table I), however
when the ligand is bound to nickel(II) the coordination compounds inhibit electron transport. The coordination compounds are more potent inhibitors on the photosynthetic electron flow than the salts, Table I.


Acknowledgements

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