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THE EFFECT OF INHIBITORS OF PLASMA MEMBRANE H^+–ATPASE AND OXIDOREDUCTASES ON NH_4^+ UPTAKE BY *PISUM ARVENSE* ROOTS

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ABSTRACT

The effect of inhibitors of plasma membrane oxidoreductases (quinacrine and dicumarol) and H^+-ATPase (dicyclohexylcarbodiimide and orthovanadate) on ammonium uptake by *Pisum arvense* seedlings and the activities of H^+-ATPase and NADH-ferricyanide oxidoreductase was investigated. The uptake solution contained 50 μM NH_4^+. In 1 h experiments, quinacrine and dicumarol depressed strongly and irreversibly the rate of NH_4^+ uptake and markedly inhibited the activity of NADH-ferricyanide oxidoreductase in the plasma membrane vesicles prepared from root cells. Simultaneously, sodium orthovanadate inhibited the activity of plasma membrane H^+-ATPase increased the rate of NH_4^+ uptake. Dicyclohexylcarbodiimide inhibited H^+-ATPase activity and increased efflux of NH_4^+ from roots to ambient solution. The results indicate on the lack of direct connection between uptake rate of 50 μM NH_4^+ and H^+-ATPase activity, and suggest that membrane redox systems play a predominant role in this process.

KEY WORDS: ammonium, uptake, inhibitors, oxidoreductase NADH-ferricyanide, H^+-ATPase.

INTRODUCTION

It has been proposed that the mechanism of NH_4^+ ions transport across plasma membrane is an electrogenic uniport (Kleiner 1981, Smith 1982). Smith et al. (1978) and Smith and Walker (1978) suggested that the transport of ammonium ions to the plant cells is mediated by specific transport system. Wang et al. (1993) found that below 1 mM external ammonium concentration, NH_4^+ influx into rice plants was due to a saturable high-affinity transport system, and above 1 mM, net fluxes of NH_4^+ was madiated by linear low-affinity transport system. It is well established that the driving force for NH_4^+ uptake is the proton electrochemical gradient across the plasma membrane (Kleiner 1981, Glass 1988). Plasma membrane H^+-ATPase is responsible for generation of proton electrochemical gradient (Serrano 1989). However, in some dicotyledonous a redox system has been found, which was able to transfer electrons from the endogenous donors to a appropriate acceptors localized out of the membrane (Möller and Crane (1990). According to Böttger et al. (1991) ferricyanide plays a role of such exogenous acceptor. Recent studies with isolated protoplasts and plasma membrane vesicles indicate that ferricyanide regulates the transport of K^+ across plasma membrane of many plant species (Rubinstein and Stern 1986, Ivankina and Novak 1988, Marré et al.1988, Roth-Bejerano et al. 1988). But even so in numerous investigations, the mechanism of NH_4^+ transport to plant cells is not yet elucidated. Thus it seems interesting to examine the effect of inhibitors of plasma membrane H^+-ATPase and oxidoreductases involved in proton and electron transport across plasma membrane, on both NH_4^+ uptake and enzymes activities in *Pisum arvense* roots.

MATERIAL AND METHODS

Plant material.

Field pea (*Pisum arvense* L. var. Nieznaniecki) seeds were germinated 2 days in darkness at 27°C. The seedlings were grown 5 days under 16 h photoperiod (10.3 Wm^{-2}) in nutrient medium wihout nitrogen (Tolley-Henry and Raper 1989) contained 0.25 mM KH_2PO_4, 0.5 mM K_2CO_3, 0.25 mM $CaSO_4$, 0.25 mM $MgSO_4$, 19 μM H_3BO_3, 10 μM ferric citrate, 3.7 μM $MnCl_2$, 0.3 mM $ZnSO_4$, 0.13 μM $CuCl_2$, 0.05 μM Na_2MoO_4 at pH 6,5.

Ammonium uptake experiments.

Groups of 17 plants without cotyledons were placed in 100 cm^3 of uptake solution contained 25 μM $(NH_4)_2SO_4$ in Tolley-Henry and Raper (1989) solution mentioned above. The solution was aerated continuously during experiments. Ammonium uptake rate was calculated from NH_4^+ depletion in nutrient solution at 15 or 30 min intervals. Ammonium was determined colorimetrically according to Hecht and Mohr (1990).

Determination of ammonium accumulated in the roots.

Two g of roots were homogenized in mortar with 10 cm^3 of distilled water. The homogenate was transferred into glass tubes and heated 10 min in 100°C. The precipitate was removed by passing the homogenate through a filter paper and ammonium content was determined in the clear solution according to McCullough (1967).

Isolation of plasma membrane vesicles.

Field pea root plasma membrane vesicles were isolated by a two-phase partitioning according to Larsson (1985) with some modifications. The roots (25 g) were homogenized in a mortar and 50 cm^3 25 mM Tris-Mes pH 7.2 with 3 mM EDTA, 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 % bovine serum albumin and 0.5 % (w/v) polyvinylpyrrolidone (PVP) was added. The homogenate was filtered through two layers of Miracloth and centrifuged at 18000 g for 10 min. The supernatant was centrifuged at 80000 g for 30 min. The pellet from the latter centrifugation was resuspended in 2 cm^3 medium containing 1 mM Tris-Mes (pH 7.2), 250 mM sucrose and 0.1 mM PMSF and layered onto two-phase gradient (2.48 g 20 % w/w dextran T 500, 1.24 g 40 % w/w polyethylene glycol 3350, 1.5 cm^3 1 M sucrose and 0.2 cm^3 117 mM KCl in 20 mM K_2HPO_4-KH_2PO_4 pH 7.8). The sample was diluted to 8 g with 20 mM K_2HPO_4-KH_2PO_4 pH 7.8, shaken virogously and centrifuged 5 min at 500 g. The upper phase was collected, diluted twice 1 mM Tris-Mes buffer pH 7.2 and centrifuged at 80 000 g for 30 min. For the measurement of H^+-ATPase activity the pellet was resuspended in 2 cm^3 1 mM Tris-Mes pH 7.2 and for NADH-ferricyanide activity in 2 cm^3 25 mM BTP-Mes pH 7.2.

Enzyme assays.

H^+-ATPase activity in plasma membrane vesicles was determined according to Gallagher and Leonard (1982) as modified by Sze (1985). The reaction medium contained (in μmoles): 33 Tris-Mes pH 7.5, 50 KCl, 2.5 $MgSO_4$, 1 NaN_3, 0.1 Na_2MoO_4, 50 $NaNO_3$, 3 ATP, 0.1 % Triton X-100 and 0.1 cm^3 of plasma membrane suspension in total volume 1 cm^3. The reaction was stopped after 10 min at 37 °C by 1 cm^3 10 % trichloracetic acid and protein precipitate was removed after centrifugation at 10 000 g for 10 min. Phosphorus was determined by the method of Ames (1966).

NADH-ferricyanide oxidoreductase activity in plasma membrane vesicles was assayed according to Kłobus and Buczek (1992). The rate of NADH oxidation was measured at 340 nm. The activities of glutamine synthetase (GS) and glutamate dehydrogenase (NADH-GDH) were determined in crude extracts after centrifugation at 20 000 g for 15 min. Crude root extract for GS was prepared in 0.05 M Tris-HCl pH 7.2 containing 10 mM $MnCl_2$ and 1 mM ditiothreitol. NADH-GDH was extracted by the method of Harper and Paulsen (1969). GS activity was measured using the transferase assay according to Hipkin and Syrret (1977) and NADH-GDH activity was assayed by the method of Duke et al. (1975).

Measurement of membrane potential difference.

Membrane potential was determined by the method described previously (Karcz and Stolarek 1988). For each treatment one intact seedling with a root length of 30-40 mm from Tolley-Henry and Raper (1989) nutrient solution was placed into a plexiglass chamber which could be perfused with the test solution. The plexiglass chamber and micromanipulator were attached to the stage of the microscope. Seedlings were adapted for 30 min to the control medium of the following composition: 1 mM KNO_3, 0.25 mM KH_2PO_4, 0.25 mM $CaSO_4$, 0.25 mM $MgSO_4$ and 10 μM ferric citrate. Membrane potentials were measured with a high-impedance electrometer amplifier (Hugo Sachs Elektronik, Type 309, Germany) and microelectrodes filled with 3 M KCl. The diameter of the microelectrode tip was ca. 1.0 μm and the tip potential did not exceed 6 mV. The microelectrodes were inserted first into an epidermal cell, then continously into underlying cortical cells between 3 and 6 mm from root apex. If no stable membrane potential difference was observed within 5-15 min, other cells or new roots were used after adaptation. The electrophysiological experiments were carried out in darkness at room temperature (24-25°C) and pH 6.5.

Protein determination.

Protein was determined by the method of Bradford (1976).

Inhibitor solutions.

Dicyclohexylcarbodiimide (DCCD) was dissolved in 0.02 cm^3 95 % ethanol and dicumarol in 0.1 cm^3 dimethyl sulphoxide. The concentration of solvents used had no effect on the activities of H^+-ATPase and NADH-ferricyanide oxidoreductase.

RESULTS

In order to investigate whether the inhibitors of plasma membrane H^+-ATPase and NADH-ferricyanide oxidoreductase affect the rate of ammonium absorption by *Pisum arvense* roots, the loss of NH_4^+ from nutrient solutions at 15 or 30 min intervals was measured. Ammonium uptake rate was significantly reduced in the presence of 50 μM DCCD (Fig. 1). The effect of 50 μM DCCD was visible after 15 min of plants treatment. The decrease of NH_4^+ uptake rate was probably caused by the increased efflux of NH_4^+ from root cells due to the action of relatively high concentration of DCCD. On the other hand, 10 μM DCCD had no effect on ammonium uptake during the first few minutes of experiments, however, after 45-50 min a slight decrease of NH_4^+ uptake was found. The seedlings treated for 1 h with different concentrations of DCCD accumulated somewhat more NH_4^+ than the control plants (Table 1), even when 200 μM DCCD was used (data not shown). The futher experiments carried out with plants pretreated for 1 h with 50 μM DCCD and then transferred to the fresh nutrient solution without inhibitor showed, that the inhibition of NH_4^+ uptake was irreversible for 1 h (Fig. 3).

The effect of 500 μM sodium orthovanadate on the rate of NH_4^+ uptake was biphasic (Fig. 2). During the first hour, orthovanadate increased NH_4^+ absorption. However, after 1 h treatment of seedlings with this inhibitor, the uptake rate of ammonium was reduced.

Subsequent experiments were undertaken to examine the effect of 500 μM sodium orthovanadate and 50 μM DCCD on the activities of GS and NADH-GDH in root cells. The results showed, that in plants treated for 1 h with orthovanadate, the activities of both enzymes were inhibited by about 52 and 37 per cent, respectively (Tab. 2). At the same time, however, 50

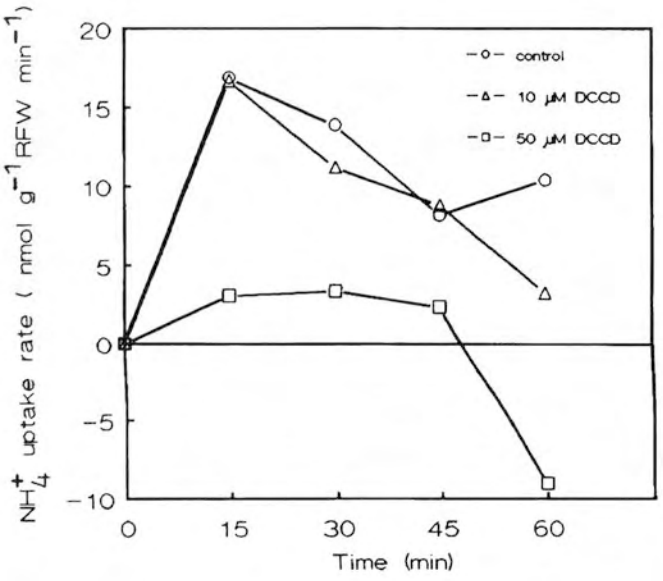

Fig. 1. Effect of DCCD on the uptake rate of NH_4^+ by *Pisum arvense*.
The plants were grown for 1 h in uptake solutions (see Material and methods) without DCCD, with 10 μM DCCD or 50 μM DCCD. Ammonium uptake rate was measured as described in Material and methods by sampling the uptake solutions every 15 minutes. The data are means of four experiments.

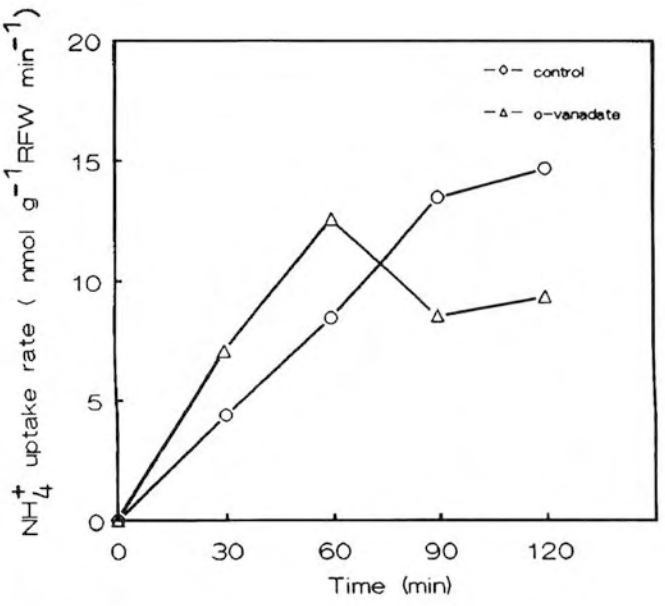

Fig. 2. Effect of sodium orthovanadate on uptake rate of NH_4^+ by *Pisum arvense*.
The plants were grown for 2 h in uptake solutions (see Material and methods) without inhibitor or with 500 μM sodium orthovanadate. Ammonium uptake rate was measured as described in Material and methods by sampling the uptake solutions every 30 minutes. The data are means of four experiments.

TABLE 1. Effect of DCCD on NH_4^+ accumulation in roots of *Pisum arvense*.

Treatment	NH_4^+ accumulated (μmoles g^{-1} f.wt. roots)
Control	0.93 ± 0.05
+ 10 μM DCCD	1.07 ± 0.03
+ 50 μM DCCD	1.28 ± 0.07

The plants were grown for 1 h in uptake solutions (see Material and methods) without DCCD or with 10 μM or 50 μM DCCD. Then the plants were harvested and NH_4^+ content in fresh matter was assayed. The data are means of four experiments.

μM DCCD increased a little GS activity and slightly decreased activity of NADH-GDH.

The effect of 250 μM quinacrine and 50 μM dicumarol on the rate of NH_4^+ uptake was shown in Fig. 3. In plants pretreated for 1 h with quinacrine or dicumarol, the absorption rate of ammonium after removal of inhibitors was markedly lower than in control ones. At the same time, the alteration in the level of ammonium accumulated in the root cells was not found (data not showed).

Both, dicumarol and quinacrine, inhibited *in vitro* the activity of NADH-ferricyanide oxidoreductase in plasma membrane vesicles isolated from Pisum arvense roots, and sligtly increased plasma membrane H^+-ATPase activity (Tab. 3). On the other hand, DCCD and sodium orthovanadate inhibited H^+-ATPase activity and orthovanadate had no inhibitory effect on the activty of NADH-ferricyanide oxidoreductase. DCCD reduced the activity of oxireductase by about 20 per cent.

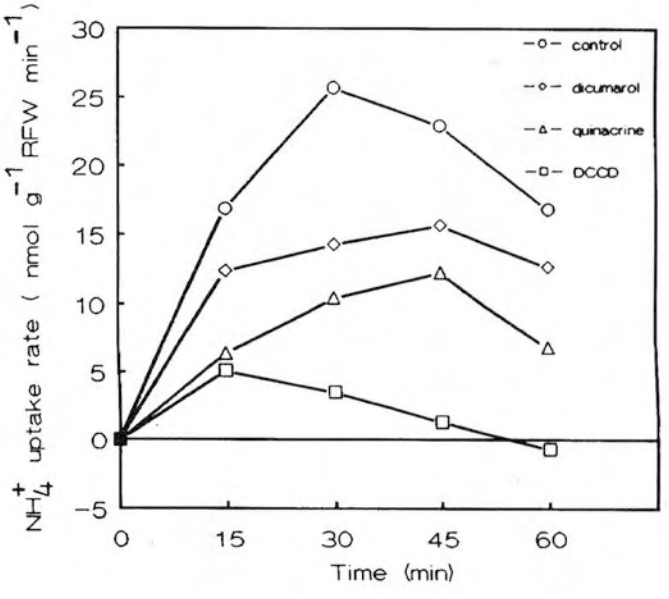

Fig. 3. Effect of preincubation of *Pisum arvense* seedlings in DCCD, quinacrine or dicumarol on the rate of NH_4^+ uptake.
The plants were preincubated for 1 h in nitrogen free solution (see Material and methods) without inhibitors (control) or with 50 μM DCCD, 250 μM quinacrine or 50 μM dicumarol. After removal of inhibitors, the plants were incubated for 1 h in ammonium solution and ammonium uptake rate was measured (Material and methods). The data are the means of four experiments.

TABLE 2. Effect of sodium orthovanadate and DCCD on glutamine synthetase (GS) and glutamine dehydrogenase (NADH-GDH) activities in *Pisum arvense* roots

Treatment	GS	NADH-GDH
	(μmoles g^{-1} f.wt. roots min^{-1})	
Control	1.42	0.125
o-Vanadate (500 μM)	0.68	0.077
DCCD (50 μM	1.86	0.106

The plants were grown for 1 h in uptake solutions (see Material and methods) without inhibitors (control) or with 50 μM DCCD or 500 μM sodium orthovanadate. The data are means of four experiments.

The average value of membrane potential difference of Pisum arvense root cells in the control medium was -72±7 mV. Quinacrine at the concentration 100 μM depolarized the root plasma membrane (Fig. 4). After 30 min of incubation of roots in the presence of quinacrine the membrane potential of root cells was equal -59±6 mV. The maximum of depolarization was reached 4 to 6 min after application of quinacrine and was 6 to 8 mV greater than that observed after 30 min.

DISCUSSION

Of the two inhibitors of plasma membrane H^+-ATPase (DCCD and orthovanadate), only DCCD distinctly reduced NH_4^+ uptake by Pisum arvense roots. The difference resulted probably from the various interaction of both inhibitors with the transport systems involved in the permeation of cations across plasma membrane.

Orthovanadate, as a very effective inhibitor of plasma membrane ATPase (O'Neill and Spanswick 1984, Sze 1985, Buczek and Sulej 1986, Serrano 1989), reduced markedly the activity of H^+-ATPase in plasma membrane vesicles from Pisum arvense root cells. However, it had no inhibitory effect on NADH-ferricyanide oxidoreductase and simultaneously on NH_4^+ uptake. The decrease in the rate of ammonium uptake

TABLE 3. Effect *in vitro* of DCCD, sodium orthovanadate, dicumarol and quinacrine on the activities of NADH-ferricyanide oxidoreductase and H^+-ATPase in plasma membrane vesicles of *Pisum arvense* roots.

Treatment	NADH-ferricyanide oxidoreductase (μmoles NADH $min^{-1}mg^{-1}$protein)	H^+-ATPase (μg P_i $min^{-1}mg^{-1}$ protein)
Control	1.14	7.63
DCCD (50 μM)	0.91	3.58
Orthovanadate (500 μM)	1.18	3.37
Dicumarol (50 μM)	0.62	8.68
Quinacrine (250 μM)	0.24	8.64

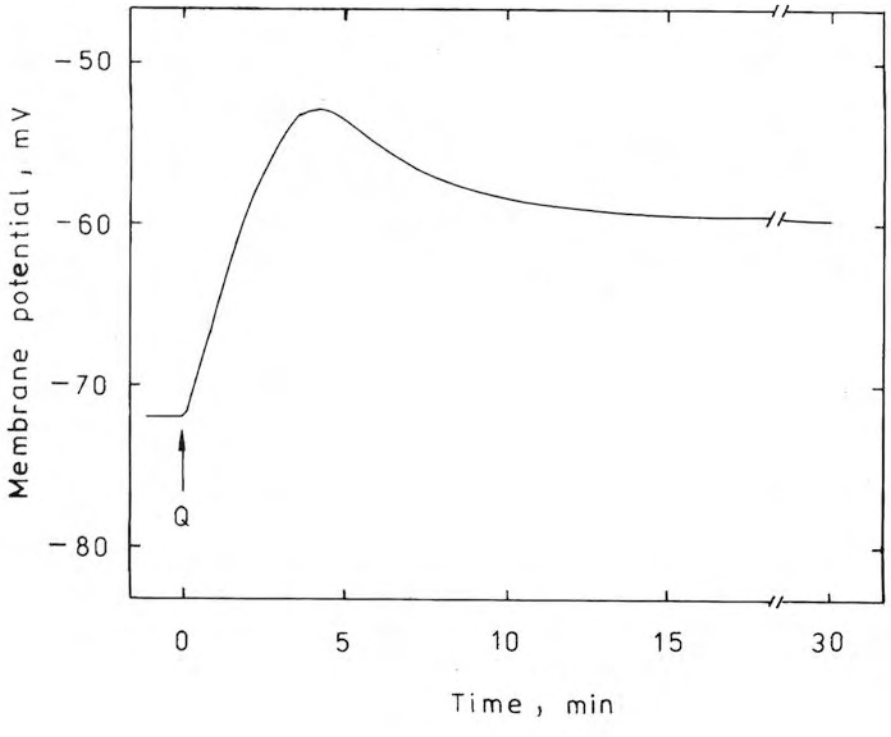

Fig. 4. Representative time course of 100 μM quinacrine (Q) effect on the cortical cell membrane potential of intact *Pisum arvense* roots. The data are means of eight mesurements.

after more than 1 h treatment of plants with orthovanadate, may be explained as a secondary effect, resulting from the reduction of activities of ammonium metabolism enzymes (GS and NADH-GDH). It appears that both GS and NADH-GDH play a certain role in NH_4^+ uptake (Kleiner 1981, Lee et al. 1992, Lee and Ayling 1993). Thus, our results suggests that the plasma membrane vanadate-sensitive H^+-ATPase does not participate in the transport of 50 μM NH_4^+ to the *Pisum arvense* root cells at least during early stages of ammonium absorption.

The results also shows that DCCD reduced markedly NH_4^+ uptake by Pisum arvense roots. The changes resulted probably from increase of ammonium efflux to ambient solution. Also Churchill and Sze (1985) have been stated, that DCCD reduced methylamine absorption (an analog of NH_4^+) in microsomal vesicles prepared from oat root cells, however, orthovanadate was uneffective. DCCD, as a not specific inhibitor of plasma membrane ATPase, easily binds to amino-, carboxyl-, sulfhydryl- and phenol groups (Solioz 1984, Hassinen and Vuokila 1993) and accelerate the depolarization of membranes (Kleiner 1981). According to Hedrich and Schroeder (1989), depolarization of membrane induces some changes in plasma membrane channels. Therefore it is not excluded, that the efflux of NH_4^+ found in our experiments was brought about by the depolarization of plasma membrane and/or induction of efflux channels rather than direct inhibition of H^+-ATPase.

Additionally, it was demonstrated that DCCD reduced by about 20 per cent NADH-ferricyanide oxidoreductase activity in plasma membrane vesicles prepared from *Pisum arvense* root cells. However, due to the slight effect of DCCD on the inhibition the enzyme activity, it is less possible, that the direct interaction of DCCD with plasma membrane oxidoreductase brought about inhibition of NH_4^+ uptake. Support for this assumption comes from observation of Lin (1984), who excluded rather a direct effect of DCCD on NADH-oxidase involved in the redox system of corn root protoplasts.

Although quinacrine and dicumarol inhibited significantly absorption of NH_4^+ by *Pisum arvense* roots there are some differences between both inhibitors. Quinacrine depolarized the plasma membrane of *Pisum arvense* roots, while dicumarol had no effect on the membrane potential of Zea mays (Döring et al. 1992 b). Quinacrine is an inhibitor of transmembrane oxidoreductases (Crane and Löw 1976, Lin 1982, Kłobus and Buczek 1992). Dicumarol has similar properties and probably binds to quinones of redox systems (Döring et al. 1992 b, Lüthje et al. 1992). The inhibitors of oxidoreductases used in our experiments reduced both the rate of NH_4^+ uptake and NADH-ferricyanide oxidoreductase activity in isolated plasma membrane vesicles. The experiments of Döring et al. (1992 a) performed on corn roots showed, that the inhibition of oxidoreductase by dicumarol was irreverible. Likewise, when dicumarol and quinacrine were removed from nutrient solution, we have found that the inhibitory effect of both compounds remains irreversible. Thus, it seems that the transport of NH_4^+ across plasma membrane of *Pisum arvense* root cells depends directly on the activity of redox system in plasma membrane.

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WPŁYW NIEKTÓRYCH INHIBITORÓW PLAZMALEMMOWEJ H^+-ATPAZY I OKSYDOREDUKTAZ NA POBIERANIE NH_4^+ PRZEZ KORZENIE *PISUM ARVENSE*

Badano wpływ inhibitorów plazmalemmowych oksydoreduktaz (kwinakryny i dikumarolu) oraz H^+-ATPazy (dicykloheksylokarbodiimidu i ortowanadanu) na pobieranie jonów amonowych przez siewki *Pisum arvense* z roztworu zawierającego 50 μM NH_4^+ a także na aktywność H^+-ATPazy i oksydoreduktazy NADH-żelazicyjanek. W jednogodzinnych doświadczeniach kwinakryna i dikumarol wyraźnie zmniejszały szybkość pobierania NH_4^+ i znacząco hamowały aktywność oksydoreduktazy NADH-żelazicyjanek w pęcherzykach plazmalemmy izolowanych z komórek korzeni. Ortowanadan hamował aktywność plazmalemmowej H^+-ATPazy i zwiększał w ciągu jednej godziny szybkość pobierania NH_4^+. Dicykloheksylokarbodiimid hamował aktywność H^+-ATPazy i zwiększał wypływ NH_4^+ do otaczającego roztworu. Uzyskane wyniki sugerują brak bezpośredniego powiązania pomiędzy szybkością pobierania NH_4^+ z roztworu o stężeniu 50 μM i aktywnością plazmalemmowej H^+-ATPazy natomiast plazmalemmowy system redoks wydaje się odgrywać w tym procesie znaczącą rolę.

SŁOWA KLUCZOWE: jony amonowe, pobieranie, inhibitory, oksydoreduktaza NADH-żelazicyjanek, H^+-ATPaza.