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Evidence for Cotransport of Nitrate and Protons in Maize Roots[1]

II. Measurement of NO_3^- and H^+ Fluxes with Ion-Selective Microelectrodes

Peter R. McClure[2], Leon V. Kochian*, Roger M. Spanswick, and Jon E. Shaff

Section of Plant Biology (P.R.M., R.M.S.) *and U.S. Plant, Soil and Nutrition Laboratory, U.S. Department of Agriculture, Agricultural Research Service,* (L. V. K., J. E. S.), *Cornell University, Ithaca, New York 14853*

ABSTRACT

We report here on an investigation of net nitrate and proton fluxes in root cells of maize (*Zea mays* L.) seedlings grown without (noninduced) and with (induced) 0.1 millimolar nitrate. A microelectrode system described previously (IA Newman, LV Kochian, MA Grusak, WJ Lucas [1987] Plant Physiol 84: 1177-1184) was utilized to quantify net ionic fluxes from the measurement of electrochemical potential gradients for NO_3^- and H^+ within the unstirred layer at the root surface. The nitrate-inducibility, pH dependence, and concentration dependence of net NO_3^- uptake correlated quite closely with the electrical response of maize roots to nitrate under the same experimental conditions (as described in PR McClure, LV Kochian, RM Spanswick, JE Shaff [1990] Plant Physiol 93: 281-289). Additionally, it was found that potential inhibitors of the plasmalemma H^+-ATPase (vandate, diethylstilbestrol), which were shown to abolish the electrical response to NO_3^- (in PR McClure, LV Kochian, RM Spanswick, JE Shaff [1990] Plant Physiol 93: 281-289), dramatically inhibited NO_3^- absorption. These results strongly indicate that the NO_3^- electrical response is due to the operation of a NO_3^- transport system in the plasmalemma of maize root cells. Furthermore, the results from the H^+-ATPase inhibitor studies indicate that the NO_3^- transport system is linked to the H^+-ATPase, presumably as a NO_3^-/H^+ symport. This is further supported by the pH response of the NO_3^- transport system (inhibition at alkaline pH values) and the change in net H^+ flux from a moderate efflux in the absence of NO_3^-, to zero net H^+ flux after exposing the maize root to exogenous nitrate. Although these results can be explained by other interpretations, the simplest model that fits both the electrical responses and the NO_3^-/H^+ flux data is a NO_3^-/H^+ symport with a $NO_3^-:H^+$ flux stoichiometry >1, whose operation results in the stimulation of the H^+-ATPase due to the influx of protons through the cotransport system.

In a companion study (14), we have characterized the electrical response of maize root cells to nitrate. Nitrate elicited a rapid, transient depolarization of the membrane potential in nitrate-induced roots. The depolarization was followed by a slower, net hyperpolarization of the membrane potential. The electrical response was nitrate-inducible and influenced by ambient pH and nitrate concentration.

Previous observations of nitrate-dependent membrane-potential depolarizations in *Lemna* fronds (17, 27) and tissues of other higher plant species (26) have been offered to support the hypothesis that nitrate transport across the plasma membrane proceeds via a NO_3^-/H^+ symport mechanism. Our observations of nitrate-dependent depolarizations of the membrane potential in maize roots suggest that such a mechanism also operates in this species.

The current research utilized a microelectrode method described previously (7, 15) to measure electrochemical potential gradients for NO_3^- and H^+ within the unstirred layer at the root surface with ion-selective microelectrodes. Net NO_3^- and H^+ fluxes were then calculated based on the measurements of ionic gradients. The objectives of the work described herein were: (a) to characterize the nitrate-inducibility, pH dependence and concentration dependence of net nitrate uptake in maize roots using nitrate-selective microelectrodes; (b) to determine if changes in the electrical response to nitrate are correlated with changes in net nitrate uptake; and (c) to examine the influence of external nitrate on the net flux of protons in maize roots.

MATERIALS AND METHODS

Plant Materials

Zea mays L. seeds (3377 Pioneer) were germinated and the seedlings were grown as described in the accompanying paper (14).

Measurement of Net Nitrate and Proton Fluxes

Liquid membrane-type neutral carrier-based H^+- and NO_3^--selective microelectrodes (tip diameter = 0.5 μm) were constructed as previously detailed (10) using Fluka H^+-selective cocktail (catalog No. 95291, Fluka Chemical Co.) and Orion nitrate cocktail (catalog No. 930701, replacement kit for nitrate macroelectrode; Orion Research, Inc.). The nitrate cocktail was graciously provided by Gordon Henriksen. For a detailed description of the construction and characterization of NO_3^--selective microelectrodes made with Orion nitrate

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[2] Present address: Syracuse Research Corporation, Merrill Lane, Syracuse, NY 13210.

cocktail, see Henriksen *et al.* (5). We quantified the net ionic fluxes associated with a small root surface area element, based on techniques developed previously (7, 10, 15), which involve the measurement of radial ion activity gradients in the unstirred layer at the root surface with ion-selective microelectrodes. These steady state gradients are the result of ion transport at the root surface (influx or efflux) and the diffusion of ions either toward or away from the root (see ref. 15 for a detailed description of the experimental procedures). Briefly, the intact seedling was housed in a Plexiglas chamber attached to the stage of an Olympus compound microscope mounted on its back on the surface of a vibration-damped table (Kinetic Systems, Inc.). The NO_3^-- and H^+-selective microelectrodes were mounted in a pressure-relieved holder on the preamplifier of a high input resistance dual electrometer (model FD 223, WP Instruments, Inc.). The preamplifier was then mounted onto a Narashige hydraulically driven micromanipulator (model MO-204, Narashige USA) which was attached to the microscope stage such that the microelectrode could be lowered vertically into the solution and reach chosen radial distances from the horizontally oriented root (usually 50 and 100 μm from the root surface).

The root and vertically positioned microelectrodes were viewed under moderate magnification (60–150×) with the Olympus microscope. To measure net NO_3/H^+ fluxes, the appropriate experimental solution was passed through the chamber until the previous solution was displaced, and then flow was stopped. The Plexiglas chamber was constructed to minimize mixing of the solution surrounding the root due to mechanical vibration and convection; we have found that steady state ion activity gradients are established at the root surface approximately 5 min after solution flow was stopped. Subsequently, NO_3^-/H^+ activities in the unstirred layer were measured at 50 and 100 μm from the root surface and the net ionic fluxes at the root surface were determined from the following equation derived from diffusion analysis of the spatial symmetry of the NO_3^- and H^+ activity gradients.

$$J_i = \frac{2\,\pi D_i(C_1 - C_2)}{\ln(R_1/R_2)}$$

where J_i is the net ionic flux (in μmol cm^{-1} s^{-1}), D_i is the self-diffusion coefficient for the ion of interest (in cm^2 s^{-1}), C_1 and C_2 are the ion activities measured at the two radial positions, and R_1 and R_2 are the respective distances from the positions where the ion activities were measured to the center of the root. The following values for diffusion coefficients (at 25°C) were used: for H^+, 9.31×10^{-5} cm^2 s^{-1} (21), and for NO_3^-, 1.92×10^{-5} cm^2 s^{-1} (20). Because of the small tip diameter of the microelectrodes, the calculated fluxes are essentially "point fluxes" that are associated with a very small root surface area element. However, the flux equation above yields a flux normalized for a 1 cm long root segment (thus the units of μmol cm^{-1} s^{-1}). Subsequently, the flux value calculated from the above equation was divided by the mass of the 1 cm segment of root (cross-sectional area of root in cm^2 × root density in g cm^{-3} × 1 cm root length) and multiplied by the appropriate units conversion factor (3600 s h^{-1}) to obtain a net flux in units of μmol g^{-1} h^{-1}. The net NO_3^- and H^+ fluxes determined in this study were measured approximately 2 cm back from the root apex. All experiments were conducted at 25°C.

RESULTS

Net nitrate uptake into roots of $CaSO_4$-grown seedlings increased during the first 2 to 3 h of exposure to 0.1 mM nitrate (Fig. 1). Uptake of nitrate during an interval commencing 160 min after first exposure was greater by approximately six-fold than uptake measured 20 min following first exposure to nitrate. This observation is in agreement with previous observations of the nitrate-induced acceleration of nitrate-uptake in maize (12). Furthermore, this time course of acceleration of nitrate uptake correlates with the developmental time course of the response of the membrane potential to nitrate in roots of identically treated seedlings as reported in the accompanying paper (14).

Net nitrate uptake by roots of nitrate-grown seedlings displayed a marked dependence upon the pH of the external solution (Fig. 2). Uptake was increasingly inhibited as the solution pH was raised from pH 4.4 to pH 8. Above pH 8, inhibition of net nitrate uptake was complete. The observation of an acidic pH optimum agrees with previous studies of the pH dependence of nitrate uptake in maize roots (13, 28). In addition, these observations correlate with the observation that in solutions of pH 8, roots of identically treated seedlings were silent electrically to nitrate (14).

The concentration dependence of net nitrate uptake by roots in the present study (Fig. 3) also was in agreement with previous studies of net nitrate uptake by maize roots (19), and, furthermore, correlates with the concentration dependence of the electrical response to nitrate in maize roots (14). In the present study, net nitrate uptake displayed an apparent K_m of 0.059 mM (Fig. 3), while the apparent K_m for the

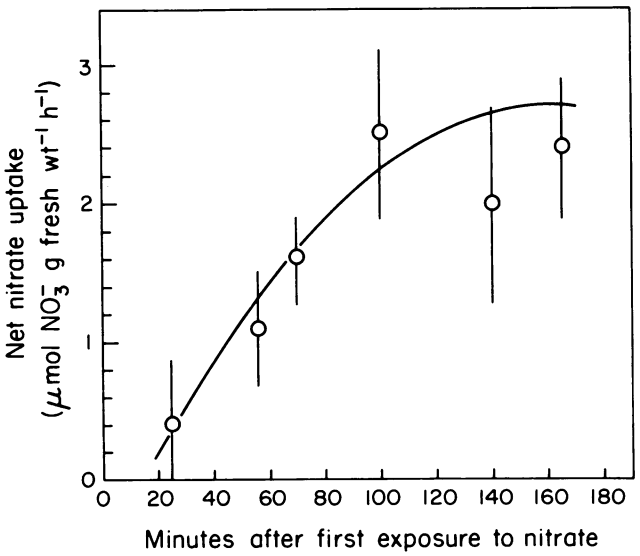

Figure 1. Time course of the acceleration of net nitrate uptake in roots of $CaSO_4$-grown maize seedlings upon initial exposure to 0.15 mM $CaSO_4$ + 0.05 mM $Ca(NO_3)_2$. Values are the means ± SEM of four replicate measurements made within 10 min intervals at times indicated. Data are from one of three experiments yielding similar results.

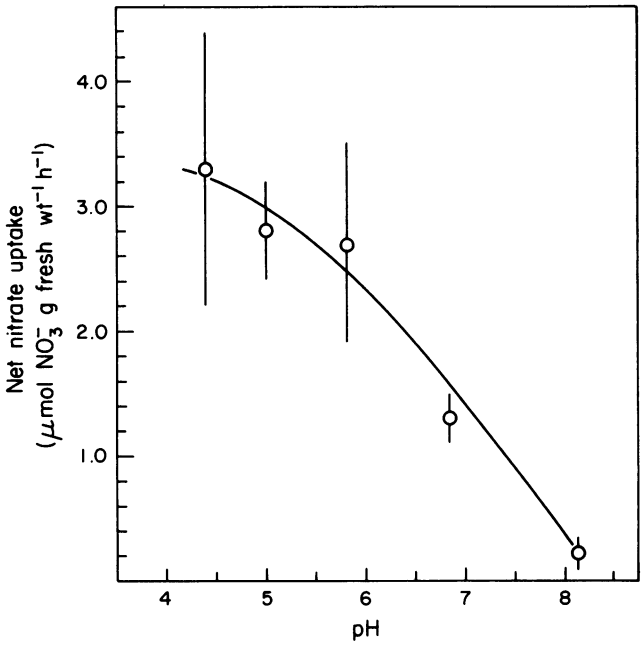

Figure 2. Dependence of net nitrate uptake upon external pH in roots of nitrate-grown maize seedlings. Values are means ± SEM for eight replicate measurements at each pH value. Solutions contained 0.15 mM $CaSO_4$ ± 0.05 mM $Ca(NO_3)_2$ and 5 mM Mes-Tris. Data are from one of four similar experiments yielding similar results.

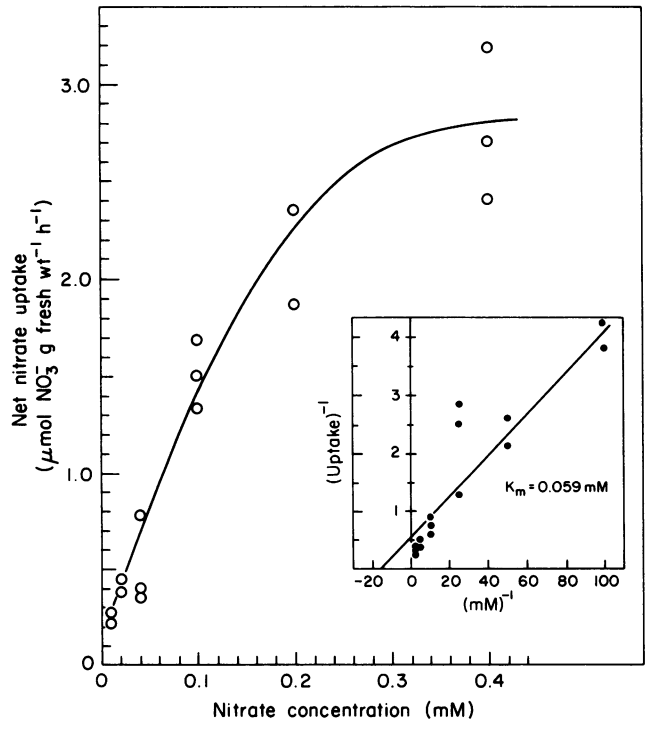

Figure 3. Concentration-dependence of net nitrate uptake in roots of nitrate-grown maize seedlings. Insert is a double-reciprocal plot of uptake data from the main panel. Data were compiled from three separate experiments.

magnitude of nitrate-dependent hyperpolarizations of the membrane potential was 0.050 mM (*cf.* Fig. 3 in ref. 14).

We reported in the companion paper (14) that treatment of maize roots with either vanadate or DES[3] dramatically inhibited the electrical response to nitrate. Each of these chemicals, which have been shown to inhibit the plasmalemma H^+-ATPase, also significantly inhibited maize root nitrate absorption (Table I). Additionally, phenylglyoxal, a chemical that reacts with arginine residues and has been shown to inhibit plant anion transport (3), abolished the nitrate electrical response (14) and net nitrate uptake in maize roots (Table I). Compounds which did not alter the electrical response to nitrate in the companion study (SITS, FC, K_2SO_4, and NH_4SO_4), likewise did not significantly alter root nitrate uptake in the present study (Table I).

When roots of either nitrate-induced or noninduced seedlings were placed in solutions containing only 0.2 mM $CaSO_4$, a net efflux of protons was routinely measured (Table II). However, when roots of either type were placed subsequently in solutions containing 0.15 mM $CaSO_4$ and 0.05 mM $Ca(NO_3)_2$, net proton efflux was decreased significantly (Table II). The relative magnitude of the decrease in net efflux of protons caused by nitrate introduction was greater in NO_3^--induced roots than in noninduced roots. Nitrate exposure

Table I. *Influence of Various Chemical Modifiers on Nitrate Uptake in Nitrate-Grown Maize Roots*

Flux values are means ± SEM of four replicate measurements from one of three experiments yielding similar results. Positive values denote a net influx, while negative values represent efflux. Control fluxes were measured in a solution consisting of 0.15 mM $CaSO_4$ + 0.05 mM $Ca(NO_3)_2$. Control fluxes were first measured 15 min after introduction of the above solution and then replicate measurements were made over a 30 min period. Control solutions were replaced with fresh solution at 15 min intervals. Control solutions were replaced with treatment solutions of identical composition except for additions as indicated. During the pretreatment periods, treatment solutions were replaced with fresh solution at 15 min intervals. Nitrate uptake in treated roots was measured over a 15 min period following the pretreatment period. Additionally, nitrate uptake under control conditions was measured over a longer time period (2 h) and was found to vary less than 20% over that period. This was done to ensure that the various treatment effects were due to the treatments and not due to other time-dependent factors (*e.g.* length of time seedling was held in the microelectrode chamber).

Treatment	Net Nitrate Uptake	
	Control	Treated
	$\mu mol\ g^{-1}\ h^{-1}$	
1 h, 0.5 mM SITS	2.4 ± 0.5	2.1 ± 0.4
1 h, 1.0 mM PGO	1.6 ± 0.2	−0.3 ± 0.2
1 h, 0.05 mM DES	2.5 ± 0.3	−0.3 ± 0.2
1 h, 0.20 mM Vanadate	2.1 ± 0.2	0.2 ± 0.2
1 h, 0.01 mM FC	1.8 ± 0.3	2.1 ± 0.5
0.5 h, 0.05 mM K_2SO_4	1.4 ± 0.2	1.6 ± 0.3
0.5 h, 0.05 mM $(NH_4)_2SO_4$	1.7 ± 0.5	1.6 ± 0.6

[3] Abbreviations: DES, diethylstilbestrol; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; FC, fusicoccin; FITC, fluorescein isothiocyanate; PGO, phenylglyoxal; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene; PMF, protonmotive force.

Table II. *Influence of Nitrate on Proton and Nitrate Fluxes in Roots of Maize Seedlings Grown With (Induced) and Without (Noninduced) 0.1 mM $Ca(NO_3)_2$*

Flux values, in units of μmol g^{-1} h^{-1}, are the means ± SEM of at least four replicate measurements from one of eight experiments with induced maize roots and one of three experiments with noninduced roots yielding similar results. Measurement of net nitrate uptake with noninduced roots were made within 30 min of first exposure to nitrate. Negative values indicate a net efflux, while positive values indicate influx.

Growth Solution	Net H^+ Efflux ($- NO_3^-$)[a]	Net H^+ Efflux ($+ 0.1$ mM NO_3^-)[b]	Net NO_3^- Uptake ($+ 0.1$ mM NO_3^-)[b]
0.2 mM $CaSO_4$ (noninduced)	−1.0 ± 0.2	−0.6 ± 0.1	0.4 ± 0.3
0.15 mM $CaSO_4$ + 0.05 mM $Ca(NO_3)_2$ (induced)	−0.8 ± 0.2	0.0 ± 0.1	2.8 ± 1.0

[a] Measurements made in 0.2 mM $CaSO_4$ solution. [b] Measurements made in 0.05 mM $Ca(NO_3)_2$ + 0.15 mM $CaSO_4$ solution.

inhibited net proton efflux by 90 ± 15% and 53 ± 12% in induced (n = 8) and noninduced (n = 3) roots, respectively.

DISCUSSION

In the first paper of this series (14), we reported that maize roots display a two-component electrical response to nitrate which consists of an initial, transient depolarization of the membrane potential, followed by a net hyperpolarization. This electrical response was: (a) nitrate-inducible; (b) inhibited by H^+-ATPase inhibitors; (c) inhibited by alkaline external pH; (d) insensitive to treatments with SITS, FC, K_2SO_4, or NH_4SO_4; and (e) dependent upon external nitrate concentration.

In the present study, we show that the nitrate-inducibility (Fig. 1), the pH dependence (Fig. 2), and the concentration dependence of net nitrate uptake (Fig. 3) by maize roots were virtually identical to responses of the membrane potential to nitrate under the same experimental conditions. Furthermore, treatment with compounds (vanadate, DES, or PGO) which inhibited the electrical response to nitrate, also inhibited net nitrate uptake (Table I). Treatment with compounds (SITS, FC, K_2SO_4, or NH_4SO_4) which did not inhibit the electrical response, did not alter net nitrate uptake (Table I). Thus, a number of characteristics of net nitrate uptake were correlated with characteristics of the electrical response to nitrate in maize seedlings. This correlation strongly suggests that the response of the membrane potential to nitrate reflects the uptake of nitrate across the plasma membrane of root cells.

Additionally, several lines of evidence presented in this paper can be used in support of the hypothesis that NO_3^- uptake is linked to the operation of the plasmalemma H^+-ATPase via a NO_3^-/H^+ symport. First, the application of H^+-ATPase inhibitors (vanadate, DES) almost completely abolished net NO_3^- uptake (Table I). Second, one would intuitively expect that an active transport system coupled to the passive influx of protons would be sensitive to changes in extracellular pH. It was observed that as the solution pH was increased to pH values near and above neutrality, a dramatic inhibition of NO_3^- uptake occurred (See Fig. 2). Although the appropriate parameters (cytoplasmic H^+ activity, membrane potential) needed to calculate the PMF were not measured in this study, the PMF was calculated from an assumed cytoplasmic pH of about 7, and previous measurements of the response of the maize root membrane potential to changes in external pH (7). It was found that the PMF was fairly constant for external solution pH values from pH 4 to 6, while for solution pH values above pH 6, the PMF declined significantly. These calculations indicate that both net NO_3^- uptake and the PMF responded quite similarly to changes in external pH. A similar relationship between PMF, amino acid uptake, and external pH was observed in rhizoid cells of *Riccia fluitans*, where amino acid/H^+ cotransport has been hypothesized to occur (6).

Finally, the simultaneous measurements of NO_3^- and H^+ fluxes yielded results consistent with the operation of a NO_3^-/H^+ cotransport system. Whenever the maize root was exposed to low levels of NO_3^- (100 μM), net H^+ efflux decreased from a moderate efflux to approximately zero net flux. This is consistent with previous reports of H^+ consumption associated with NO_3^- uptake (4, 24), and also transient alkalinization of the medium during the operation of sugar/H^+ cotransport systems (8, 9). It was not possible to measure a net H^+ influx when 100 μM NO_3^- was applied to maize roots; at best, the net H^+ flux was reduced to zero. This may reflect the relatively low level of NO_3^- used, and the potentially complex situation that could exist between H^+ influx via the symport mechanism, a subsequent stimulation of the H^+-ATPase by changes in membrane potential and cytoplasmic H^+ activity, and other ion transport processes (see, for example, refs. 6 and 9). In this study, when higher concentration of NO_3^- were used (5–10 mM), a net alkalinization (H^+ influx) was often observed (data not shown).

In cyanobacteria and algae, the presence of ambient ammonium causes a short-term inhibition of net nitrate uptake (25). In higher plants, however, reported effects of NH_4^+ on nitrate uptake are quite variable, ranging from a transient stimulation (1), to no effect (16), to a total inhibition of net nitrate uptake (2). This variability is not well understood, and cannot be explained by species differences alone. In maize, for example, reports of an inhibitory NH_4^+ effect (11) are countered by reports that ambient NH_4^+ exerts very little influence on net nitrate uptake (18, 23).

In our experiments with maize seedlings, the presence of ammonium (0.1 mM) did not significantly inhibit net nitrate uptake from ambient solutions containing 0.1 mM nitrate (Table I). Likewise, treatment of similar maize seedlings with

ammonium did not alter the response of root-cell-membrane potentials to nitrate (14). These data are in agreement with reports that NH_4^+ does not inhibit nitrate uptake into maize roots (18, 23), but, upon first consideration, appear to conflict with the reports of Mackown *et al.* (11) and Rufty *et al.* (22). Both of these previous studies reported that NH_4^+ significantly inhibited nitrate uptake in maize seedlings; however, in both studies the inhibition was not significant until after 2 h of exposure to NH_4^+. Our investigations of NH_4^+-effects on nitrate uptake (Table I) and on the electrical response to nitrate (14) never lasted more than 1 h (including the time of pretreatment). Thus it seems likely that, if we had treated the roots in our experiments with NH_4^+ for a longer period of time, we may have noted an inhibitory effect on both the electrical response and net nitrate uptake. Confirmation of this suggestion awaits further experimentation.

In conclusion, the results presented here, based on the simultaneous measurement of NO_3^- and H^+ fluxes at the maize root surface, indicate that: (a) the nitrate electrical response detailed in the first paper is the result of NO_3^- uptake into maize root cells, (b) the NO_3^- transport system is linked to the operation of the plasmalemma H^+-ATPase, and (c) the link between NO_3^- absorption and the H^+-ATPase appears to be due to a H^+-NO_3^- symport which has a H^+:NO_3^- flux stoichiometry > 1 and may indirectly stimulate the activity of the H^+ pump.

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