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Characteristics of Injury and Recovery of Net NO_3^- Transport of Barley Seedlings from Treatments of NaCl

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ABSTRACT

The nature of the injury and recovery of nitrate uptake (net uptake) from NaCl stress in young barley (*Hordeum vulgare* L, var CM 72) seedlings was investigated. Nitrate uptake was inhibited rapidly by NaCl, within 1 minute after exposure to 200 millimolar NaCl. The duration of exposure to saline conditions determined the time of recovery of NO_3^- uptake from NaCl stress. Recovery was dependent on the presence of NO_3^- and was inhibited by cycloheximide, 6-methylpurine, and cerulenin, respective inhibitors of protein, RNA, and sterol/fatty acid synthesis. These inhibitors also prevented the induction of the NO_3^- uptake system in uninduced seedlings. Uninduced seedlings exhibited endogenous NO_3^- transport activity that appeared to be constitutive. This constitutive activity was also inhibited by NaCl. Recovery of constitutive NO_3^- uptake did not require the presence of NO_3^-.

Inhibition of ion uptake, Ca^{2+}, K^+, Pi, and NO_3^- by plants growing under saline conditions has been reported by many investigators (1, 3, 8, 9, 15, 16, 20). We recently showed in short term experiments that salinity inhibited net NO_3 uptake by barley seedlings but had little effect on its reduction (1) and that Ca^{2+} increased NO_3^- uptake under saline conditions (20). Little is known about the mechanisms of salt inhibition of ion uptake. Displacement of Ca^{2+} from cell membranes by Na^+ under saline conditions is thought to disrupt membrane integrity, resulting in alterations in ion transport ability (3, 4). Inhibition of Pi uptake by barley roots was correlated with the loss of proteins from the roots during osmotic shock in ice-cold saline solutions (15).

Nitrate uptake represents a unique means to study the influences of salt on membrane transport activities since the transporter is inducible by NO_3^- (1, 7, 11, 18, 20), is presumably located in the plasma membrane (2), apparently requires RNA and protein synthesis (11), and net uptake is inhibited by salt. Little is known concerning (a) how rapidly injury occurs to the root NO_3^- uptake system with an inhibitory concentration of salt in the nutrient medium, and (b) the characteristics of recovery after salt is removed. We show the interaction of NaCl concentrations and time on the recovery of NO_3^- uptake from salt damage. In addition, the effects of inhibitors of RNA, protein, fatty acid, and sterol synthesis on reinduction and recovery of uptake are shown.

MATERIALS AND METHODS

Seedling Growth. Barley (*Hordeum vulgare* L., var CM 72) seedlings were grown hydroponically as described before (1, 20). Seedlings were grown for the first 7 d in continuous darkness in aerated 0.2 mM $CaSO_4$ and then were transferred to one-quarter strength Hoagland solution lacking N (10) and placed in a controlled environment growth chamber. In the N-free Hoagland solution, K_2HPO_4, KH_2PO_4, $CaSO_4$, and $Ca(H_2PO_4)_2$ were substituted for KNO_3 and $Ca(NO_3)_2$. To minimize the effects of rhythms, the seedlings were grown under continuous light for the next 3 d at 25°C and 60 to 65% RH. The photon flux density at the seedling canopy was 400 μE m^{-2} s^{-1} and was supplied by cool white fluorescent and incandescent lamps. In specified experiments, after 2 d in continuous light, the seedlings were transferred to one-quarter strength N-free Hoagland solution containing 1 mM KNO_3 for 24 h to fully induce the NO_3^- uptake system (induced seedlings) (1, 7, 11, 20).

Effect of NaCl on NO_3^- Uptake. *Induced seedlings.* All pretreatment and uptake solutions used in the study contained one-quarter strength N-free Hoagland (10) solution with 1 mM Mes (pH 6.2) and were aerated continuously. Groups of 10 plants (10 d old) weighing about 4 g total were placed in 50 ml of uptake solutions containing 0.7 mM KNO_3, with increasing concentrations of NaCl between 0 and 250 mM for the specified times indicated in the figure legends. Uptake rates were determined by sampling the uptake solutions at the specified times. Each experiment was replicated at least three times.

Uninduced Seedlings. Groups of 10 uninduced seedlings were transferred to 50 ml of N-free uptake solutions with increasing concentrations of NaCl between 0 and 200 mM for 5 min. The roots were then rinsed five times for a total time of 3 min with 100 ml of the same solution without NaCl and were transferred to 50 ml of salt-free uptake solution containing 0.1 mM KNO_3. Nitrate uptake was determined by sampling the solutions every 5 min for 20 min.

Reversibility of Salt Effects. Groups of 10 induced seedlings were placed in 50 ml of uptake solutions containing 0.7 mM KNO_3 to determine initial uptake rates. As a control, one set of 10 seedlings remained in these solutions to determine their activity throughout the time course of the experiment. Measurements were made every 15 min for the first 3 or 4 h and after 11 h. The uptake solution was changed after each determination. After 1 h, the other sets of 10 induced seedlings were transferred to 400 ml of uptake solution with 200 mM NaCl for 5 min or 1 or 3 h. After the respective salt treatments, the seedlings were rinsed as described above. The seedlings were then transferred to 50 ml of salt-free uptake solution, and the rate of NO_3^- uptake was determined at the times specified above.

Effect of Ca^{2+} on Reversibility of Salt Effects. Groups of 10 induced seedlings were placed in uptake solutions of 50 ml with 200 mM NaCl and 0.7 mM NO_3^-. Uptake rates were determined in the presence of salt by sampling the solutions every 5 min. After 1 h, the seedlings were rinsed for 3 min as described above and then placed in 500 ml of modified one-quarter strength

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Hoagland solution with 1 mM KNO_3 and 0, 0.65, or 3.0 mM $CaCl_2$. In the modified Hoagland solution, K_2SO_4 and KH_2PO_4 were substituted for $CaSO_4$ and $Ca(H_2PO_4)_2$. After 3 h, the seedlings were transferred to 50 ml of uptake solution with 0.7 mM KNO_3. Uptake rates were determined by sampling solutions every 5 min for 1 h. Uptake solutions were changed after each determination.

Effect of NO_3^- on Reversibility of Salt Effects. *Induced Seedlings.* Groups of 10 induced seedlings were placed in 50 ml of uptake solution with 0.7 mM KNO_3 with and without 200 mM NaCl to determine initial uptake rates. The seedlings were then rinsed as above with NO_3^--free uptake solution and transferred to 500 ml of salt-free uptake solution with 0 or 0.7 mM KNO_3. After 3 h the seedlings were transferred to 50 ml of fresh salt-free uptake solution with 0.7 mM KNO_3 and were sampled every 5 min for 1 h to determine uptake rates.

Uninduced Seedlings. Uninduced seedlings were stressed with 200 mM NaCl for 5 min and rinsed as above with uptake solutions lacking NO_3^-. After the 3-min rinse, the seedlings were transferred to 500 ml of NO_3^--free uptake solution. Groups of 10 seedlings were removed after 1, 2, and 3 h and were transferred to 50 ml of fresh salt-free uptake solution with 0.1 mM KNO_3. Uptake was determined by sampling the solutions every 5 min for 20 min.

Effects of CHI,[2] 6-Methylpurine, and Cerulenin. Groups of 10 seedlings were placed in 50 ml of uptake solutions containing 0.7 mM KNO_3 for 1 h to determine the initial uptake rates. Uptake rates were determined by sampling solutions every 15 min. Uptake solutions were changed after each determination. Two sets of seedlings were then transferred to uptake solutions containing 200 mM NaCl. After 1 h, the salt-stressed seedlings were rinsed as described above and transferred to uptake solutions with and without CHI (2 μg/ml), or 6-methylpurine (0.5 mM), or cerulenin (2 μg/ml). A set of control seedlings was also transferred to fresh uptake solution with the corresponding inhibitor.

For the induction experiments, groups of 10 uninduced seedlings were transferred to 50 ml of nutrient solution containing 0.7 mM KNO_3 with and without 2 μg/ml of cerulenin, or 2 μg/ml of CHI, or 0.5 mM 6-methylpurine. Uptake was determined by sampling solutions every 15 min, and rates were calculated for each sampling interval.

Nitrate Analysis. Nitrate was determined by HPLC as described by Goyal and Huffaker (6). All results are reported on a fresh weight basis.

RESULTS

Nitrate uptake by barley seedlings was not affected by 50 and 100 mM NaCl, whereas higher concentrations—150, 200, and 250 mM—decreased NO_3^- uptake 30, 50, and 65%, respectively (Fig. 1). Since 200 mM NaCl inhibited NO_3^- uptake by about 50% in short term studies, this concentration was used in all subsequent experiments.

The rate of NO_3^- uptake was inhibited rapidly by NaCl, within 1 min after the seedlings were placed in salt solution (Fig. 2A). The rate of NO_3^- uptake further decreased to 50% of the control rate during the first 5 min in NaCl but decreased only another 10% during a subsequent 10 h in NaCl (Fig. 2B).

The time required for recovery of NO_3^- transport from NaCl inhibition (after removal of salt from the nutrient solution) was similar (about 3 h) for 5 and 60 min of salt treatment (Fig. 3). Recovery was exponential with a rapid phase about 1 h after the removal of salt. More serious damage resulted between 1 and 3 h, which, although still reversible, required 12 h for recovery. A lag period of about 2 h was observed before a linear rate of recovery occurred.

The presence of NO_3^- in the substrate solution was required for the recovery of NO_3^- transport activity by the salt-stressed seedlings (Table I) while it was not essential for maintenance of NO_3^- uptake in the control seedlings during the 3 h time course. The presence of Ca^{2+} in the substrate solution was not required for the recovery of NO_3^- transport from salt stress (Table II).

The recovery of NO_3^- uptake from salt damage was blocked by CHI, 6-methylpurine, and cerulenin, respective inhibitors of

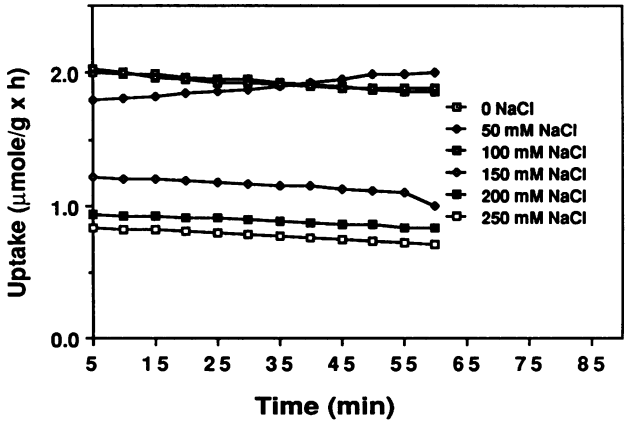

FIG. 1. Effect of NaCl on NO_3^- uptake in NO_3^--induced barley seedlings. See "Materials and Methods" for procedures.

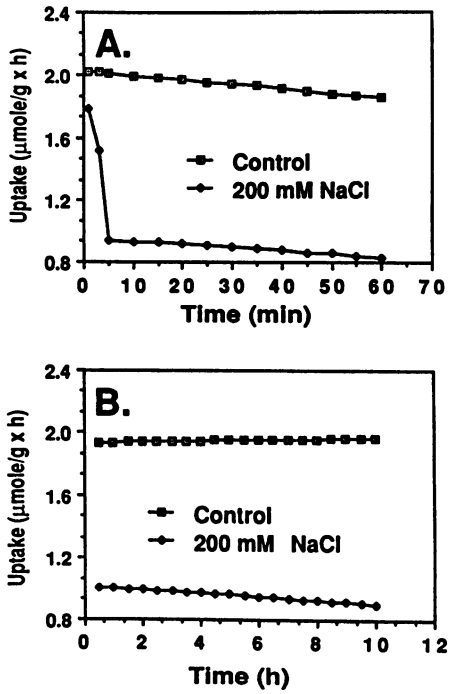

FIG. 2. Inhibition of NO_3^- transport by NaCl as a function of time in induced barley seedlings. Groups of 10 seedlings were transferred to 50 ml of solutions containing 0.7 mM KNO_3 with or without 200 mM NaCl. Plants were treated with NaCl for 1 or 3 min, rinsed five times for a total time of 3 min with 100 ml of salt-free uptake solution, and then transferred to 50 ml of salt-free uptake solution. Uptake was determined by sampling solutions 5 min after transfer to the salt-free solutions. For the subsequent time points, seedlings were maintained in the salt solutions for the duration of the uptake experiments. Uptake was determined by sampling every 5 min in the 1-h time course and every 30 min for the 10-h time course.

[2] Abbreviations: CHI, cycloheximide; HMG, 3-hydroxy-3-methylglutaryl; ACP, acyl carrier protein.

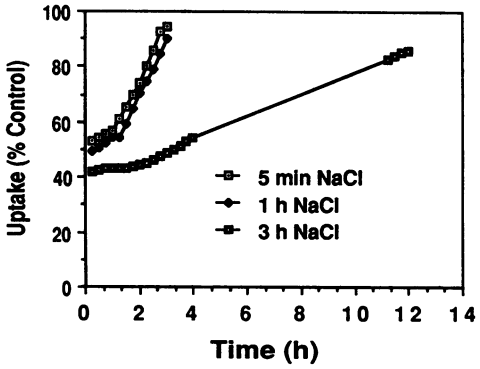

FIG. 3. Recovery of NO_3^- transport activity by induced seedlings from NaCl treatment. See "Materials and Methods" for procedures. The NO_3^- uptake rate for the control (100%) was quite constant over the time course and averaged 2.08 μmol/g × h.

Table I. *Effect of NO_3^- on the Recovery of NO_3^- Uptake from NaCl Stress*

See "Materials and Methods" for procedures.

Treatment		NO_3^- Uptake Rate	
Initial	Final	Initial	Final
		μmol/g × h	
KNO_3	KNO_3	2.5	2.6
KNO_3		2.4	2.4
KNO_3 + NaCl	KNO_3	1.1	2.2
KNO_3 + NaCl		1.0	1.2

Table II. *Effect of Ca^{2+} on the Recovery of NO_3^- Uptake from Salt Stress.*

See "Materials and Methods" for procedures.

Ca^{2+} Concentration	NO_3^- Uptake Rate	
	Initial	Final
mM	μmol/g × h	
0	0.75	2.01
0.65	0.70	1.95
3.0	0.73	2.05

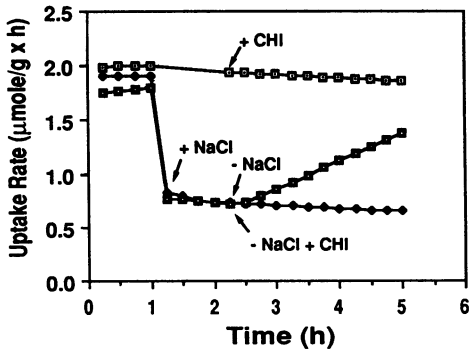

FIG. 4. Effect of CHI on the recovery of NO_3^- transport activity from NaCl treatment by induced seedlings. See "Materials and Methods" for procedures.

protein, RNA, and sterol/fatty acid synthesis (Figs. 4 to 6) (11, 13, 17, 19). In the absence of the inhibitors, uptake rates of NaCl-stressed seedlings recovered to over 80% of the control in 5 h. The inhibitors had little effect on NO_3^- uptake by the control seedlings.

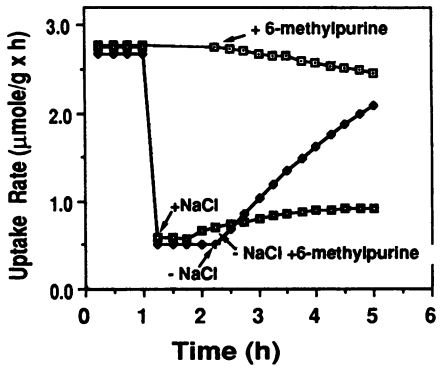

FIG. 5. Effect of 6-methylpurine on the recovery of nitrate uptake from NaCl stress by induced seedlings. See "Materials and Methods" for procedures.

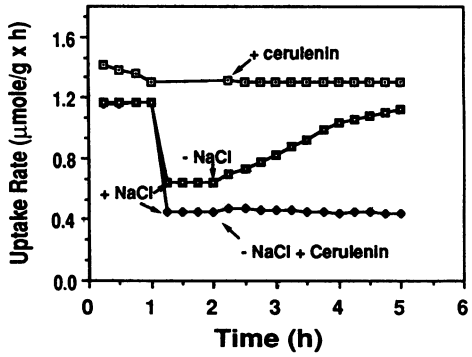

FIG. 6. Effect of cerulenin on the recovery of NO_3^- uptake from NaCl stress by induced seedlings. Experimental details are in "Materials and Methods."

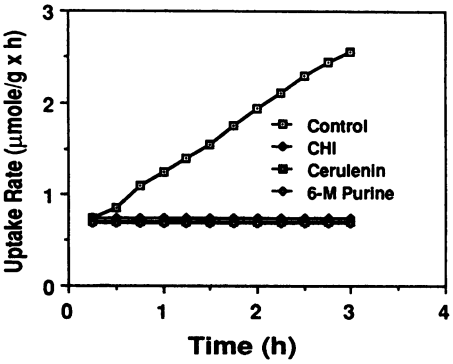

FIG. 7. Effect of CHI, 6-methylpurine, and cerulenin on the induction of NO_3^- transport in uninduced seedlings. Experimental details are in "Materials and Methods."

Each of the inhibitors was also tested for its effect on the initial induction of the NO_3^- transporter in control plants (Fig. 7). All inhibitors completely inhibited the induction of the inducible NO_3^- uptake system.

The effect of salt stress on endogenous (possibly constitutive) NO_3^- transport in uninduced seedlings is shown in Figure 8. Constitutive NO_3^- uptake was not affected by a 5-min exposure to 50 or 100 mM NaCl, whereas 150 and 200 mM NaCl decreased NO_3^- uptake 70%. Similar to the induced seedlings, recovery from a 5-min 200 mM salt treatment required 3 h (Fig. 9). Unlike preinduced seedlings, however, recovery of NO_3^- transport to near the level of the control did not require the presence of NO_3^- in the external solution.

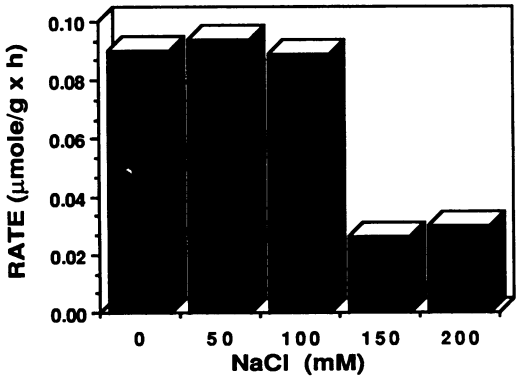

FIG. 8. Effect of NaCl on NO_3^- uptake in uninduced seedlings. See "Materials and Methods" for procedures.

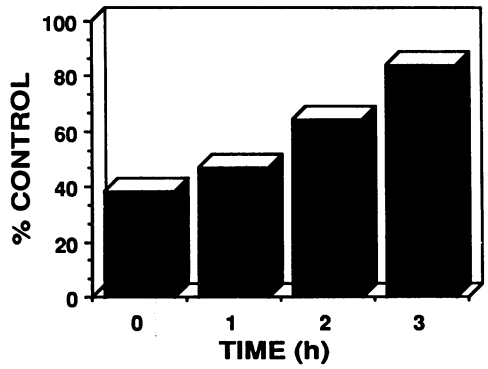

FIG. 9. Recovery of NO_3^- transport from salt stress by uninduced seedlings. See "Materials and Methods" for procedures. The control (non-salt-stressed) rate was 0.133 μmol/g × h (100%).

DISCUSSION

Effect of NaCl Concentration. Net NO_3^- uptake by barley seedlings was quite tolerant to salt during the 12-h time course of the experiments, showing little effect of salinity until an ambient concentration of 150 mM NaCl was reached. Several phases of inhibition of NO_3^- transport by salt were detected. Inhibition was detected rapidly; within 1 min after the roots were put into a 200 mM NaCl solution, NO_3^- uptake decreased 10%, 25% at 3 min, and 50% of the control after 5 min (Fig. 2A). During a subsequent 10 h in salt (Fig. 2B), uptake of NO_3^- decreased only another 10%; hence, 80% of the total inhibition occurred during the first 5 min in salt.

Recovery of Uptake. The type of damage sustained between 5 and 60 min of salt treatment appeared to be similar, *i.e.* the damage was not very severe, since total recovery occurred in 3 h in either case (Fig. 3). However, more serious damage resulted from the salt treatment between 1 and 3 h, which, although still reversible, required 12 h for recovery and had an increased lag period before recovery of uptake began. Inhibition was a function of concentration × time between 1 and 5 min but not between 5 and 60 min since inhibition was almost constant. Between 60 min and the next 9 h in salt, uptake decreased only another 10% but the time required for the recovery of uptake increased 3-fold. Perhaps the ability to reinduce the transporter complex suffered as time in salt increased beyond 1 h.

The inhibition of NO_3^- uptake within 1 min by salt and its slow recovery (several hours) indicate several possible mechanisms of injury: the NO_3^- transport system may have been damaged. This is indicated by the observation that recovery of uptake from salt injury required the presence of the inducer, NO_3^- (Table I), and may have required both RNA and protein synthesis (Figs. 4 and 5). It has been shown (7) that the initial induction of the NO_3^- transport system requires NO_3^- (11, 18) and takes about 8 h for full induction. Use of inhibitors indicates also that the induction may require synthesis of both protein and RNA (11) (Fig. 7). Cerulenin, an inhibitor of HMG-CoA synthetase (17) and β-keto-acyl ACP synthetase (19), inhibited both recovery of uptake from salt injury (Fig. 6) and the initial induction of the NO_3^- uptake system in control plants (Fig. 7), indicating the need for congruent lipid and/or sterol biosynthesis. Cerulenin had no effect on the induction of nitrate reductase in detached barley leaves, suggesting that protein synthesis was not affected by the inhibitor (data not shown). Another possible component of decreased NO_3^- uptake in the presence of salt may be an increase in an efflux component. Efflux of NO_3^- has been detected for barley and wheat plants (5, 12, 14). That recovery of net uptake may involve replacement of injured components appears feasible since it seems doubtful that enough new roots would develop in 3 h sufficient to recover almost full capacity of NO_3^- uptake.

Maas et al. (15) showed that cold, osmotically shocked segments of barley roots released protein into the external solution at similar concentrations of salt at which Pi uptake was reversibly inhibited. Treatments of CHI and puromycin inhibited the recovery of Pi uptake, indicating a requirement for protein synthesis. They proposed that peripheral binding proteins that may be required for uptake of Pi were stripped off the plasma membrane by salt and that recovery required their resynthesis.

The NO_3^- uptake activity seen in the presence of the protein and RNA synthesis inhibitors was due to an endogenous NO_3^- transporter that appears to be constitutive. It is present in barley roots that have not been exposed to NO_3^- and is apparently not under rapid turnover since its activity did not decrease in the presence of either inhibitor. Although the endogenous transporter is inhibited by NaCl (Fig. 8) similarly to the inducible transporter, it is apparently under different control from the inducible uptake system since it does not require an NO_3^- pretreatment period for activity (Fig. 8) nor does it require NO_3^- in the external solution during recovery from salt stress (Fig. 9).

Effect of Ca^{2+}. It has been proposed that in the presence of salt, roots are damaged by the displacement of Ca^{2+} from the plasma membrane by Na^+ (3). Ward *et al.* (20) showed that Ca^{2+} efficiently protected NO_3^- uptake in the presence of salt but had no effect when supplied either before or after the salt stress. Ca^{2+} also decreased the length of the lag phase for induction of the NO_3^- transporter. On this basis, the results in Table II were unexpected: recovery from the salt treatments did not require the presence of Ca^{2+} in the uptake solution. Since the roots were rinsed briefly with a one-quarter strength Hoagland solution after removal from salt, it is possible that sufficient Ca^{2+} was either absorbed or adsorbed to supply the Ca^{2+} requirement.

In summary, net NO_3^- uptake was rapidly decreased by the addition of salt to the substrate solution. After removal of salt, the recovery of inducible NO_3^- uptake required NO_3^- and was inhibited by CHI, 6-methylpurine, and cerulenin. Salt also decreased the endogenous NO_3^- uptake system, but recovery was not dependent on the presence of NO_3^- in the uptake solution. These results indicate that recovery may have required a resynthesis of the NO_3^- transporter(s) and a concomitant synthesis of lipids and/or sterols.

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