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EFFECT OF SODIUM CHLORIDE ON THE NITRATE REDUCTASE OF *SUAEDA MARITIMA* VAR. *MACROCARPA*

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Key Word Index—*Suaeda maritima* var. *macrocarpa*; Chenopodiaceae; nitrogen metabolism; nitrate reductase; effect of sodium chloride.

Abstract—The nitrate reductase (NR) activity extracted from *Suaeda maritima* is reduced by half in the presence of 0.1 M sodium chloride. This effect of sodium chloride is the result of an uncompetitive inhibition of the enzyme for nitrate and NADH. Addition of salt in the growth medium enhances the synthesis of NR without modifying its affinity constants. There is a fundamental difference between the long-term and short-term actions of sodium chloride. In *Suaeda*, the activity of NR must be the resultant of the effect of sodium chloride which inhibits the catalytic activity of the enzyme and stimulates the synthesis of the enzymatic protein.

INTRODUCTION

The halophily of *Suaeda macrocarpa* is expressed by an activation of nitrogen biosyntheses when sodium chloride is added to the growth medium [1]. Salinities from 130 to 170 mM favour the growth of this plant and coincide with a high production of intermediate nitrogenous metabolites [2, 3]. Simultaneously there is an important demand for mineral nitrogen [4] with an activation of the assimilation of the ammonium ion. We have shown that the optimal salinity for the development of *Suaeda* (129 mM NaCl) increases the incorporation of the ammonium ion by stimulating the leaf pathway of the enzymatic couple glutamine synthetase–glutamate synthase [5] while the glutamate dehydrogenase pathway, located in the roots essentially, is clearly depressed [6].

The key enzyme of the enzymatic sequence leading to the production of ammonium ions is nitrate reductase (NR EC 1.6.6.1) which also plays a primary role in the assimilation of mineral nitrogen. Its activity is generally inhibited by extreme external conditions (water loss, high temperature) [7–9]. Moreover, addition of sodium chloride to the nutrient medium of glycophytes such as wheat [10] or cotton [11] is followed by a decrease of NR activity while the opposite effect is observed with the two halophytes, *Salicornia europaea* [12] and *Suaeda maritima* var. *macrocarpa* [13].

In this work we report the localization and the levels of the potential activity of NR in *Suaeda maritima* var. *macrocarpa* grown at different salinities. The effects of sodium chloride on the kinetic characteristics of the enzyme are detailed by comparison between the long term action of the salt (addition in the growth medium) and short term action (addition *in vitro*).

RESULTS

Long term action of sodium chloride

Localization of NR in the plant. The NR of *Suaeda maritima* is located almost exclusively in the shoots, the activity of the roots amounting at most to 1% of the total activity on a fr. wt basis of the entire plant. It is possible to detect a root NR, but its activity always remains below 5% of that in the leaf tissue. The salinity of the medium and the age of the plants do not alter these results in contrast to what is observed with *Cochlearia anglica* [14]. Most of the nitrates are reduced in the aerial organs of this halophyte, when the plants are grown without salt, while in the presence of salt, the roots contribute an important proportion of the NR.

Activity of NR. The origin of the plants affects the activity of NR. Table 1 shows that NR activity is increased by *ca* 50% after 25 days growth when the salinity shifts from 0 to 129 mM. The beneficial effect of sodium chloride on the intensification of the NR is increased by the length of time the plant is in contact with salt since the activity of the enzyme increases *ca* 20% between days 25 and 45 of growth. At both intervals, the level of NR in the plants grown on a medium highly enriched with salt (393 mM) is relatively close to the level calculated for the optimal salinity. Compared to the result obtained with the salt-enriched medium, the *Suaeda* grown in a sodium chloride-free medium exhibit a low NR activity, which decreases *ca* 70% after 45 days of culture. The depressive influence of the lack of salt on NR activity can be explained by the enzyme's instability *in vivo* [15]. NR is a highly labile enzyme, its activity can be modified by numerous factors affecting the plant's physical environment. In the case of the halophyte

Table 1. Effects of sodium chloride concentration in the nutrient medium on the specific activity of NR in the aerial organs of *Suaeda* after 25 and 45 days of growth

Days of growth	Specific activities Salinity of the nutrient medium (mM NaCl) 0	129	393
25	2.70	4.2	3.15
45	0.87	4.9	3.20

Sp. acts. = nmol nitrite formed/min/mg protein.

Suaeda maritima var. *macrocarpa*, absence of sodium chloride calls such unfavourable conditions which can be compared to the physical alterations due to extreme environmental situations [7–9] (water loss, high temperature).

Michaelis constants of NR. The different sodium chloride concentrations in the nutrient medium (0–393 mM) do not affect the values of the K_m calculated for NADH (Fig. 1, curves A) and nitrates (Fig. 1, curves B). The K_ms are respectively 5×10^{-5} M for the cofactor and $0.9–1.1 \times 10^{-4}$ M for the substrate. These values are similar to those obtained for glycophytes [16] measured in extracts made in the presence of 5 mM cysteine as in our study. Beevers *et al.* [16] have clearly shown that the affinity constants will vary with the amount of sulfhydryl groups carried by the enzyme. This also explains why Stewart *et al.* [17] working with *Suaeda* found an affinity for nitrates somewhat lower than in our case because they incubated their enzyme without cysteine.

Short-term action of sodium chloride

Activity of NR. In order to study the action of sodium chloride *in situ* on NR activity, its effect was followed *in vitro* on a purified extract originating from plants grown under optimal conditions (129 mM NaCl). Both assay mediums tested (see Experimental) received 12.5–300 mM sodium chloride. The pH of 7.5 chosen in this study was unaffected by the salt increments. Figure 2 shows that NR activity is similar in both types of buffer (phosphate and Tris–HCl 0.1 M). In each case activity is reduced to 50% with 100 mM sodium chloride. The cationic strength of the assay medium is then 110 mM (mainly from Na^+) with Tris–HCl buffer and over 210 mM ($Na^+ + K^+$) with phosphate buffer. This shows that inhibition is due to sodium chloride and not to high ($Na^+ + K^+$) ion concentration. This result does not exclude the possibility of an inhibitory effect of potassium chloride since Heimer showed that the NR of the halophilic alga *Dunaliella* was equally sensitive to potassium chloride and to sodium chloride [18]. The physiologically significant point remains the fact that sodium chloride is inhibitory with Na^+ at much lower than the concentrations in the cytoplasm of marsh grown *Suaeda*. The extracts obtained from *Suaeda* grown without sodium chloride or under supra-optimal growth conditions (393 mM) show the same response to the qualitative effect of sodium chloride *in vitro*. The NR of the salt-tolerant species *Salicornia europaea* is also affected *in vitro*, its activity decreasing

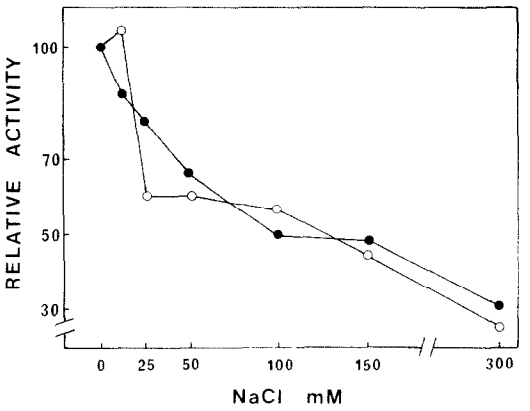

Fig. 2. Effect of sodium chloride concentration in the reaction mixture on the activity of NR extracted from aerial organs of *Suaeda* grown under optimal conditions. Results expressed as percentages of the activity measured without addition of salt in the reaction mixture. (●) potassium phosphate buffer 0.1 M; (○) Tris–HCl buffer 0.1 M.

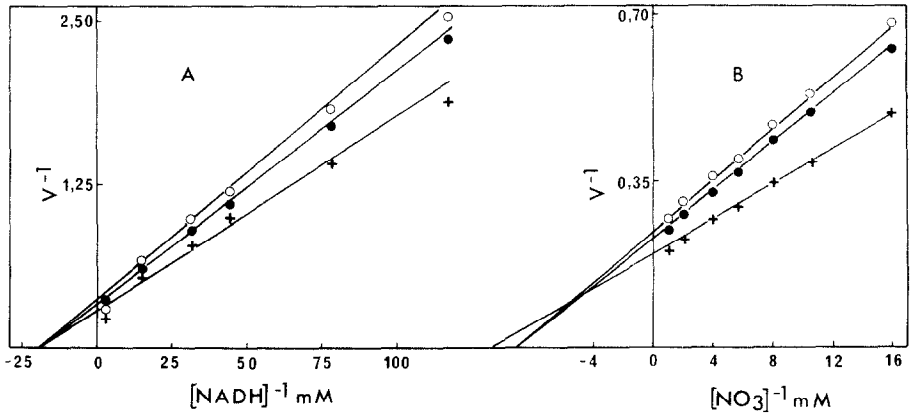

Fig. 1. Determination of Michaelis constants (K_m) by the Lineweaver–Burk method for NADH (A) and NO_3^- (B). Aerial organs of *Suaeda* grown without sodium chloride (+) and in the presence of 129 mM (○) and 393 mM (●) sodium chloride, V = nmol NO_2^- formed/min.

progressively with sodium chloride concentrations over 25 mM [12].

Michaelis constants of the NR. Action of sodium chloride on the kinetics of NR was also studied *in vitro* (Figs. 3 and 4). The Lineweaver–Burk plot shows the uncompetitive nature of the inhibition caused by sodium chloride at all nitrate concentrations tested. A similar result is achieved when the amount of NADH is reduced. The importance of the salt's inhibitory action on the reduction of nitrates is not dependent on the concentration of substrate or cofactor. In both cases, in the presence of uniform amounts of the inhibitor (100 or 200 mM) the straight lines obtained are in fact parallel to those found without sodium chloride. This inhibition reduces the affinity constants of NR for NO_3^- and NADH in similar proportions and also the maximum velocity of the enzymatic reaction. In contrast to many enzymes from halophytes and particularly dehydrogenases [6, 19, 20] where the inhibition due to sodium chloride is of the competitive type, the diminution of the

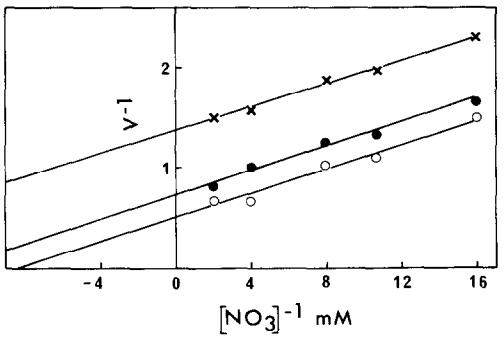

Fig. 3. Effect of sodium chloride, 0 (○); 100 (●) and 200 mM (×) in the reaction mixture at different NO_3^- concentrations on the kinetic characteristics of NR extracted from aerial organs of *Suaeda* grown under optimal salinity conditions (129 mM NaCl).

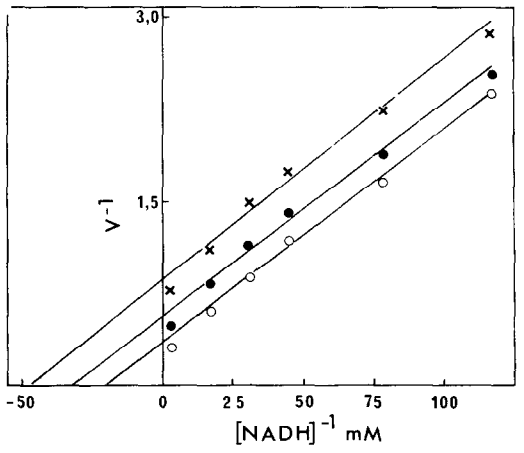

Fig. 4. Effect of sodium chloride, 0 (○); 100 (●) and 200 mM (×) in the reaction mixture at different NADH concentrations on the kinetic characteristics of NR extracted from aerial organs of *Suaeda* grown under optimal salinity conditions (129 mM NaCl).

catalytic potential of the NR in *Suaeda* cannot be explained in terms of a fixation of sodium chloride on the free enzyme. By combining exclusively with the enzyme–substrate complex, the sodium chloride creates a new inactive enzyme–substrate–sodium chloride unable to provide the normal product [21].

DISCUSSION

Optimal growth of *Suaeda macrocarpa* requires *ca* 129 mM sodium chloride in the growth medium. Reduction of NO_3^- is most rapid and its incorporation into protein highest for this concentration of sodium chloride as one of us demonstrated [4] using ^{15}N label. The results obtained here indicate that the NR located essentially in the aerial tissues shows in a salt-enriched medium and at both intervals (25 and 45 day's growth), an activity potential sufficiently high to provide this important demand for reduced nitrogen. The plants grown without salt always display the lowest activity. The latter is less than 20% of the NR activity of plants grown for 45 days at the optimal salinity. This stimulating effect of salt *in vivo* is reported in other halophytes such as *Salicornia europaea* [12] and *Cochlearia anglica* [14] while an adverse effect is noted in glycophytes with the exception of *Phaseolus aconitifolius* [22]. These results therefore show that plants grown under unfavourable saline conditions (absence of sodium chloride for halophytes, addition of sodium chloride for glycophytes) always show the lowest levels of NR activity. NR, with a very rapid turnover [23] is an example of an enzyme which is very responsive to environmental conditions. For instance absence of water supply in barley [24] and wheat [25] seedlings or addition of salt in the growth medium [10] will lower by *ca* 6% the tissue hydration and reduce NR activity. Absence of sodium chloride will lead to similar results in the case of *Suaeda*: a 6% drop in hydration of the tissues and a decrease in the activity of the enzyme. The beneficial effect of salt on the NR activity of *Suaeda* could be explained partly by the preservation of a low degree of hydration. The hydration factor however cannot be taken into account alone, since NR activity at the optimal salinity increases with time without any change in the hydration of the tissue. Sodium chloride can interfere in the control of the synthesis of the enzyme as was shown by Oji and Izawa [26] in rice. We have reported a similar action *in vivo* for glutamine synthase [27] and glutamate dehydrogenase [6] of *Suaeda*.

The action of sodium chloride in the long term does not alter the affinity constants of NR. They remain very similar to those of glycophytes whatever the salinity of the growth medium. This conclusion which is drawn from purified extracts must however be tempered by the effects of sodium chloride observed *in vitro*.

When added to the assay mixture the salt strongly inhibits NR since its activity is reduced by half when the sodium chloride concentration reaches 100 mM. This result agrees with the well-established fact that most enzymes extracted from halophytes show a similar *in vitro* sodium chloride sensitivity [19]. This inhibitory effect of sodium chloride in the case of *Suaeda* is better understood when the modifications of the affinity constants of NR are analysed. *In vitro*

the salt induces an uncompetitive inhibition for all substrate or cofactor concentrations tested, lowering both the K_m and V_{max} of the enzymatic reaction. Sodium chloride does not stabilize itself on the free NR but on the NR–substrate complex.

This study shows that a fundamental difference exists between the long-term and short-term action of sodium chloride. Located in leaf cytoplasm [28], the NR is in contact with sodium chloride amounts equal to or greater than 100 mM [29]. Its activity can therefore be reduced by half, pre-supposing that the conditions of the *in vitro* study (NO_3^- and NADH values) are compatible with the range of intracellular concentrations. In *Suaeda* the NR activity must then be the resultant of the effect of sodium chloride which inhibits the catalytic activity of the enzyme and at the same time stimulates the synthesis of the enzymatic protein.

EXPERIMENTAL

Seeds of *Suaeda maritima* (L.) Dum. var. *macrocarpa* Moq. were germinated for 12 days with a temp. cycle of 12 hr at 5° and 12 hr at 25°. The seedlings were transplanted and growth conditions were as described previously [30], in the absence or presence of 129 mM and 393 mM NaCl.

Nitrate (KNO_3) and ammonia [$(NH_4)_2SO_4$] were present in a concn ratio equal to 3.7. The roots and aerial parts were analysed after 25 and 45 days of growth. The fresh tissues, *ca* 3 g, were ground at 2° in a Pi buffer (0.1 M) with 5 mM cysteine, 1 mM EDTA, pH 7.5. After centrifugation at 25 000 *g* for 20 min, the supernatant was filtered and desalted on a Sephadex G-25 column equilibrated with Pi buffer (0.1 M) containing 5 mM cysteine, pH 7.5. The NaCl present in the extracts was therefore eliminated. Protein in the enzyme preparation was estimated according to the method of ref. [31]. The cultures were at different salinities and each analysis was repeated at least ×3.

Sp. act. and kinetic characteristics of NR (EC 1.6.6.1) were assayed using NADH as cofactor in a reaction mixture containing in a final vol. of 2 ml, 20 μmol KNO_3; 4 nmol NH_2OH, HCl; 0.64 μmol NADH; 0.012 μmol FAD; 0.2 mmol Pi buffer; final pH 7.5. The assay medium already contained over 110 mM cations mainly from KPi buffer. For this reason the *in vitro* study of the effects of NaCl was conducted replacing the Pi buffer by a Tris–HCl 0.1 M buffer in the extraction, purification and incubation media. In this case the cationic strength of the medium is lowered to 10 mM (presence of KNO_3, 0.01 M as substrate) before adding NaCl. Addition of the enzymatic extract marked the beginning of the reaction. NO_2^- formation was stopped after 20 min incubation at 30° by adding 0.2 ml 0.1 M ZnOAc according to the method of ref. [32]. NO_2^- ions were estimated by *A* at 520 nm after addition of nitrite diazotation reactants. Sp. acts. were expressed as nmol NO_2 formed/min/mg protein. The interval between preparation of the extract and NR analysis never exceeded 1 hr.

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