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Nitrate Reductase Activity and Polyribosomal Content of Corn (*Zea mays* L.) Having Low Leaf Water Potentials[1]

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ABSTRACT

Desiccation of 8- to 13-day-old seedlings, achieved by withholding nutrient solution from the vermiculite root medium, caused a reduction in nitrate reductase activity of the leaf tissue. Activity declined when leaf water potentials decreased below −2 bars and was 25% of the control at a leaf water potential of −13 bars. Experiments were conducted to determine whether the decrease in nitrate reductase activity was due to reduced levels of nitrate in the tissue, direct inactivation of the enzyme by low leaf water potentials, or to changes in rates of synthesis or decay of the enzyme.

Although tissue nitrate content decreased with the onset of desiccation, it did not continue to decline with tissue desiccation and loss of enzyme activity. Nitrate reductase activity recovered when the plants were rewatered with nitrate-free medium, suggesting that the nitrate in the plant was adequate for high nitrate reductase activity. The rate of decay of nitrate reductase activity from desiccated tissue was essentially identical to that of the control, *in vivo* or *in vitro*, regardless of the rapidity of desiccation of the tissue. Direct inactivation of the enzyme by the low water potentials was not detected. Polyribosomal content of the tissue declined with the decrease in water potential, prior to the decline in nitrate reductase activity. Changes in ribosomal profiles occurred during desiccation, regardless of whether the tissue had been excised or not and whether desiccation was rapid or slow. Reduction in polyribosomal content did not appear to be associated with changes in ribonuclease activity. Nitrate reductase activity and the polyribosomal content of the tissue recovered upon rewatering, following the recovery in water potential. The increase in polyribosomal content preceded the increase in nitrate reductase activity. Recovery of enzyme activity was prevented by cycloheximide.

Based on these results, it appears that nitrate reductase activity was affected primarily by a decrease in the rate of enzyme synthesis at low leaf water potentials.

A number of metabolic changes occur when leaf water potentials decrease in plants. Among these are large changes in the activity of certain enzymes (1, 12, 7, 22). One of the most severely affected is nitrate reductase which has been shown to decline to 42% of the controls in barley leaves having a Ψ_w[2] of about -11 bars (12) and to 50% of the controls in corn seedlings having a water deficit of 10% (1). This large effect of desiccation on NR is not surprising, since the enzyme is very labile (13, 17) and is affected by many environmental factors (3, 8, 15, 26).

It has been suggested (1) that the depressing effect of low Ψ_w on enzyme activity might be due to decreases in the rate of protein synthesis (2, 5, 18) or to increases in the rate of enzyme degradation. Since NR is substrate inducible (3, 13, 27), the decline in activity with desiccation might reflect a decrease in nitrate content of the tissue. Low Ψ_w might also cause changes in enzyme characteristics resulting in differential inactivation which would be dependent on the specific enzyme. The objective of this work was to test these alternatives with nitrate reductase in corn during desiccation which was achieved by withholding water.

MATERIALS AND METHODS

Corn plants (*Zea mays* L., Oh 43 × B 14) were grown in a constant environment chamber (temperature: 31 C day and 23 C night; relative humidity: 45%; light intensity: 1.9 cal cm^{-2} min^{-1}; photoperiod: 14 hr). Thirty seeds were planted 2.5 cm below the surface in 1 liter of vermiculite that was held in a plastic container (11 × 11 × 16 cm). The container had numerous holes in the bottom for subirrigation and drainage. The seeds and plants were subirrigated daily by immersion in a larger container filled with a full strength, modified Hoagland's nutrient solution.

Desiccation. In certain experiments, desiccation was initiated by terminating subirrigation of the seedlings 6 days after planting. In other experiments, shoots of 8- or 9-day-old seedlings were excised and allowed to desiccate rapidly in the air under continuous light for 1 to 1.5 hr. The shoots were then transferred to a Plexiglas humid chamber (33 C) to minimize further reduction in Ψ_w.

Measurement of Leaf Water Potential. Leaf water potentials were measured with a thermocouple psychrometer as described by Boyer (6) in the same tissue used for determination of NR activity.

Extraction and Assay of Nitrate Reductase Activity and Tissue Nitrate. Fresh leaf material (0.5 g) was ground in a Ten Brock homogenizer with six volumes of extraction medium (3). The homogenate was centrifuged at 29,000*g* for 15 min, and the supernatant was used for assay of both NR activity (17) and tissue nitrate content (16).

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[2] Abbreviations: NR: nitrate reductase; ψ_w: leaf water potential.

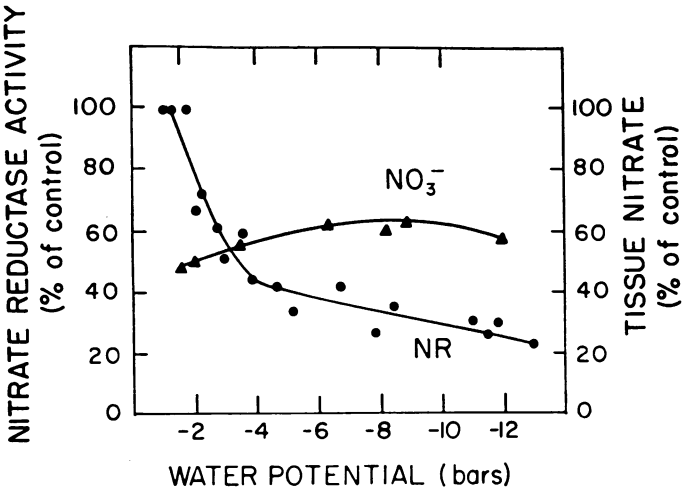

FIG. 1. Effect of decreasing leaf water potentials on NR activity and tissue NO_3^- content in 8- to 13-day-old seedlings. Plants were desiccated by withholding nutrient solution from the root medium. Desiccation was started 6 days after planting. The controls were kept well watered with nutrient solution throughout the experiment. These data represent the results of several experiments. In a typical experiment the initial level of NR and NO_3^- were 144 μmoles of NO_2^-/g dry weight·hr and 5910 μg/g dry weight, respectively. In the control plants NR values of 103, 96, and 87 μmoles of NO_2^-/g dry weight·hr and NO_3^- content of 5890, 5420, and 4150 μg/g dry weight were determined at the time when the leaf water potentials of the desiccated plants were −2.2, −5.1 and −13 bars.

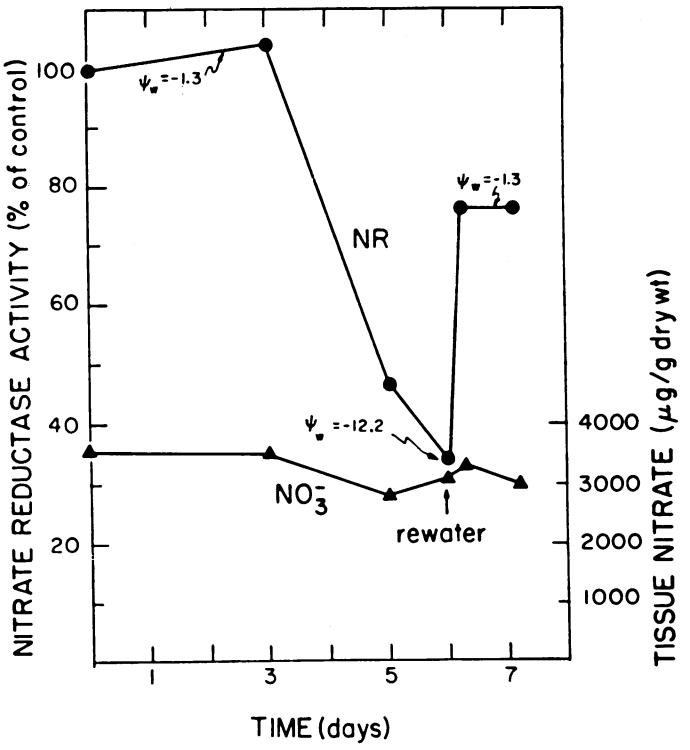

FIG. 2. Changs in NR activity (●) and tissue NO_3^- content (▲) of intact corn seedlings during desiccation and, 6 and 24 hr after rewatering with nitrate-free solution. Water was withheld 2 days prior to the first sampling (day 1). Nitrate content and NR activity of control (well watered) plants at day 1 were 8750 μg/g dry weight and 957 μmoles of NO_2^-/g dry weight·hr, respectively.

Extraction and Assay of Ribonuclease. Fresh leaf tissue (0.7 g) was ground in a Ten Brock homogenizer with 2.1 ml of 50 mM citrate buffer (pH 5.0) containing 0.5 M KCl. The homogenate was centrifuged at 1,000g for 10 min, and the supernatant was used for assay of RNase activity (25).

Measurement of Ribosomal Profiles. Ribosomes were extracted using a modification of the method described by Travis *et al.* (20). Fresh leaf material (3 g for 8- to 9-day-old seedlings, 5 g for older seedlings) was cut into small sections and immediately frozen in liquid nitrogen. Subsequently, the tissue was placed in a mortar at Dry Ice temperature and pulverized. The powder was transferred to an appropriate tube containing 15 ml of cold grinding medium containing diethyl pyrocarbonate (20) and homogenized in a Willems Polytron PT 20 at high speed for 2 to 3 sec. The homogenate was filtered through one layer of Miracloth and centrifuged at 30,000g for 15 min. Seven ml of the supernatant were layered on 5 ml of 1.5 M sucrose and centrifuged for 1 hr at 270,000g (Spinco 65 rotor). This large ratio of supernatant volume to sucrose volume was used because the interface between the two solutions held up a certain amount of ribosomes, especially monoribosomes. Consequently, the larger the volume of supernatant, the greater was the proportion of ribosomes pelleted and the more accurately the pellet reflected the entire ribosomal profile of the supernatant. The pellets obtained were resuspended in 0.6 ml of suspension medium (20), and 0.5 ml of this suspension was layered on 24 ml of a 10 to 34% linear sucrose density gradient and centrifuged at 59,000g (Spinco SW 25) for 2 hr. The gradients were fractionated in an ISCO Model D density gradient fractionator and recording spectrophotometer (Model UA-2). Absorbance was measured at 254 nm.

The polyribosomal content of the tissue was calculated from the areas of polyribosomes (P), subunits (S) and monoribosomes (M), according to P/(P+S+M).

RESULTS AND DISCUSSION

Tissue Nitrate and Nitrate Reductase Activity during Desiccation. Nitrate reductase activity decreased in intact corn seedlings that were desiccated by withholding nutrient solution from the root medium from day 6 to day 13 after planting (Fig. 1). The Ψ_w of the desiccated seedlings changed from −1.3 to −13 bars during this time. The ψ_w of the controls remained relatively steady throughout the experiment, between −1.0 and −1.8 bars. At Ψ_w below −2 bars, NR activity in the desiccated seedlings was always lower than in the control. At a Ψ_w of −13 bars, the activity in the desiccated seedlings was 23% of that in the well watered controls. This effect of moderate desiccation on NR activity agrees with that reported for barley (12) and corn (1).

In contrast to the changes in NR activity, the nitrate content of the tissue decreased markedly with the onset of desiccation, but then increased from 48 to 55 to 60% of the control value with the continuing decrease in Ψ_w (Fig. 1). This suggests that there was no correlation between the changes in NR activity and nitrate content.

Rewatering intact seedlings that had been desiccated for 7 days (Ψ_w = −12.2 bars) with nitrate-free solution resulted in a complete recovery of Ψ_w (−1.3 bars) after 6 hr. During this same period, NR activity changed from 34% of the control after desiccation to 76% of the control after recovery, and remained at the latter level for 24 hr (Fig. 2). There was no significant change in the nitrate content of the tissue during this time (Fig. 2). These results indicate that although the amount of nitrate in the desiccated tissue was considerably less than in the control, it was still sufficient for high NR

activity. Huffaker *et al.* (12) also concluded that the decrease in nitrate levels could not account for the reduction in NR.

There is one possibility the results do not rule out. Evidence has been presented that nitrate is compartmentalized in cultured tobacco cells (9). Removal of the exogenous nitrate supply from the cells caused a decrease in NR activity in spite of the high concentrations of nitrate that were present presumably in inactive pools in the cells. Similar observations have been reported for the roots of corn seedlings (14). It would be possible then that low Ψ_w could have prevented the movement of nitrate to the induction site, whereas rewatering could have made nitrate available again for the induction of nitrate reductase.

Rates of Decay of NR Activity *In Vitro* and *In Vivo* after Desiccation. Since the nitrate content of the tissue did not appear to account for the reduction in NR activity at low Ψ_w, experiments were done to determine whether faster decay of the enzyme occurred in desiccated tissue. Rates of decay of NR after tissue desiccation were measured *in vivo* and *in vitro* and compared with the rates of decay of the enzyme in well watered controls.

The rate of decay of NR extracted from excised shoots that had been desiccated for 1 hr was essentially the same as that from well watered seedlings or from excised well watered shoots (Fig. 3A). The decline was linear with time and showed a half-life of 2 hr for NR.

An experiment was also conducted with extracts from intact seedlings that had been desiccated slowly for 8 days by withholding nutrient solution from the root medium. The loss of NR activity was slower in extracts from desiccated seedlings than in those from well watered controls of the same age (Fig. 3B). The half-life of NR in the desiccated seedlings was 4.5 hr, but it was only 2.8 hr in the controls. Apparently, there was an increase in stability of the enzyme with long term desiccation. The decay rates of NR for well watered seedlings were similar to those reported by Warner *et al.* (24).

The *in vivo* rate of decay of NR in shoots excised from 10-day-old seedlings and desiccated in the air for 1 hr was compared with the rate of decay of NR in shoots excised from similar seedlings that had been pretreated for 3 hr with cycloheximide (50 μg/ml) but not desiccated. Cycloheximide has been shown to reduce the synthesis of the enzyme without affecting its decay (17). All excised shoots were kept in a humid chamber under continuous light for 30 hr and sampled every few hours. The Ψ_w of the desiccated seedlings was -10.5 bars after 1 hr of desiccation, but continued to decrease slowly in the humid chamber until the end of the experiment when the Ψ_w of the desiccated seedlings was -22 bars (Fig. 4, inset). There was no significant difference between the rate of decay of NR *in vivo* in desiccated seedlings and the rate in well watered seedlings in which the synthesis of the enzyme had been inhibited with cycloheximide (Fig. 4). Calculations from these data show an average half-life of NR of 4.25 hr which is similar to data reported by Schrader *et al.* (17).

The *in vitro* and *in vivo* studies of NR decay indicate that the decrease in the NR activity that occurs at low Ψ_w was not due to an increase in the rate of decay of the enzyme, regardless of the rate at which desiccation was carried out.

Inactivation of NR during Desiccation. Since the rate of decay of NR did not increase during desiccation, it was possible that there was inactivation of the enzyme at low leaf Ψ_w. Two different approaches were used to investigate this possibility. Herrera *et al.* (10) have recently reported that NR from *Chlamydomonas reinherdi* inactivated by ammonia could be immediately reactivated *in vitro* by addition of small amounts of ferricyanide. K. Jetschmann, L. P. Solomonson, and B.

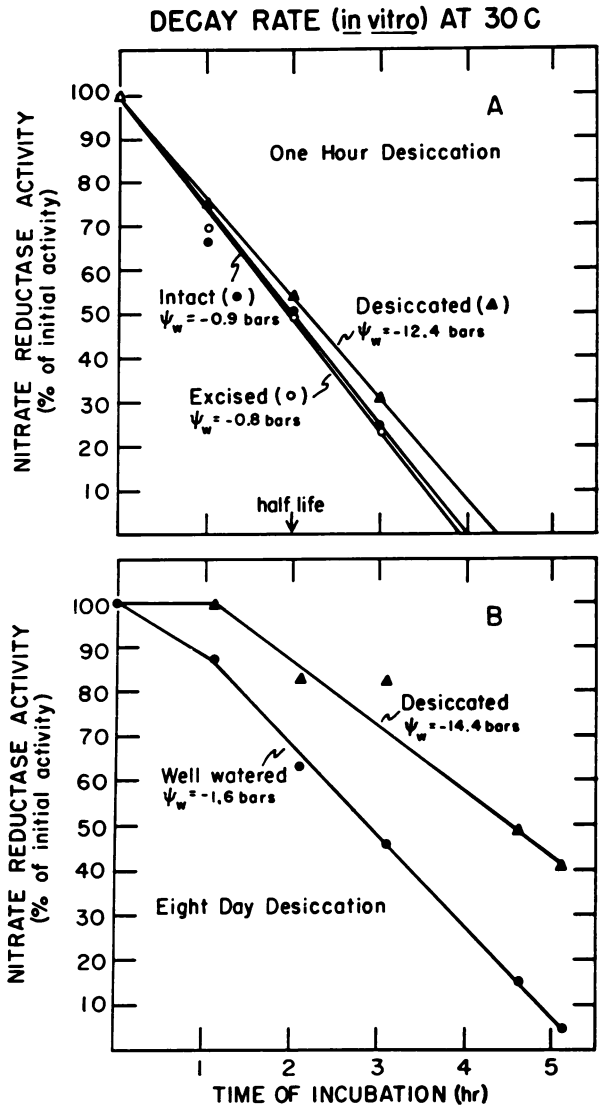

FIG. 3. *In vitro* rates of decay of NR activity in extracts from corn seedlings. The enzyme was extracted from desiccated seedlings and well watered controls and the crude extracts were incubated in a water bath at 30 C for 6 hr and assayed for NR activity every hour. A: Extracts from 8-day-old seedlings: intact, well watered controls (●); excised, well water controls (○); excised, desiccated for 1 hr (▲). Initial activities were 126, 114, and 96 μmoles of NO_2^-/g dry weight·hr, respectively. (B) Extracts from 13-day-old seedlings: intact, well watered controls (●); intact, desiccated for 8 days (▲). Initial activities were 63 and 11 μmoles of NO_2^-/g dry weight·hr, respectively.

Vennesland (private communication) have also been able to activate inactive forms of NR in *Chlorella* by incubation with ferricyanide. However, all attempts to reactivate NR extracted from desiccated plants by incubation with ferricyanide were unsuccessful.

The possibility of direct inactivation of the enzyme by the low leaf Ψ_w was also investigated by rapidly desiccating excised shoots from 8-day-old seedlings for 1.5 hr and then placing the shoots in a humid chamber. NR activity was unaffected after 1.5 hr, even though the Ψ_w of the tissue had decreased from -1.5 to about -12 bars (Figs. 5, A and B). Subsequently, NR activity in the desiccated seedlings declined slowly and after 4 hr was 52% of that in the well watered controls. Since

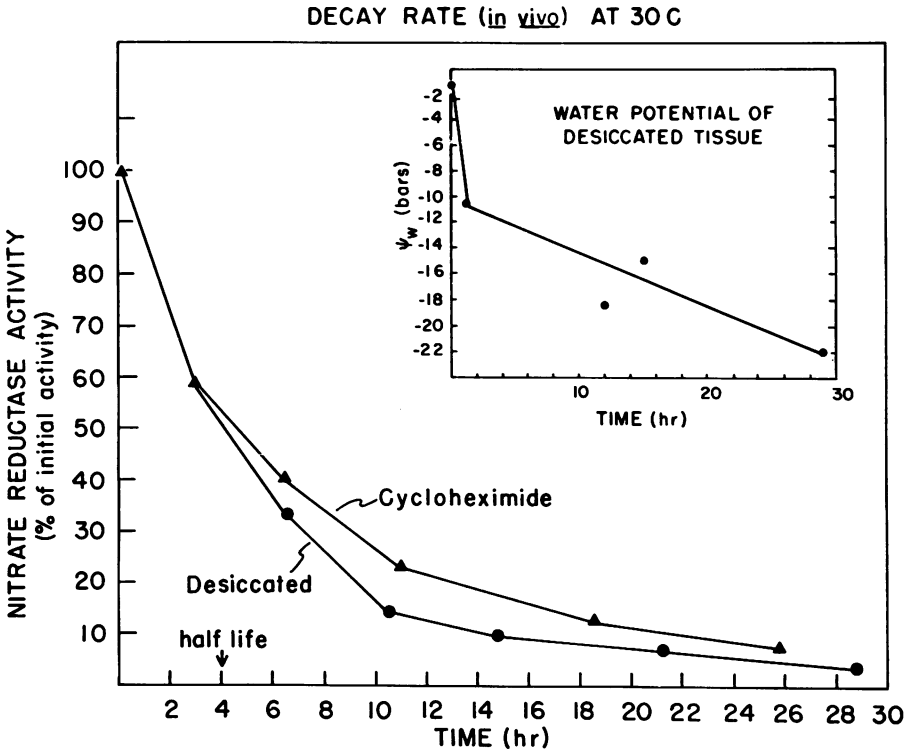

FIG. 4. *In vivo* rates of decay of NR activity in desiccated and well watered 10-day-old corn seedlings at 30 C. Synthesis of NR in the wel watered seedlings was prevented by preincubation with cycloheximide (50 μg/ml) for 3 hr. Initial activity was 79 μmoles of NO_2^-/g dry weight·hr

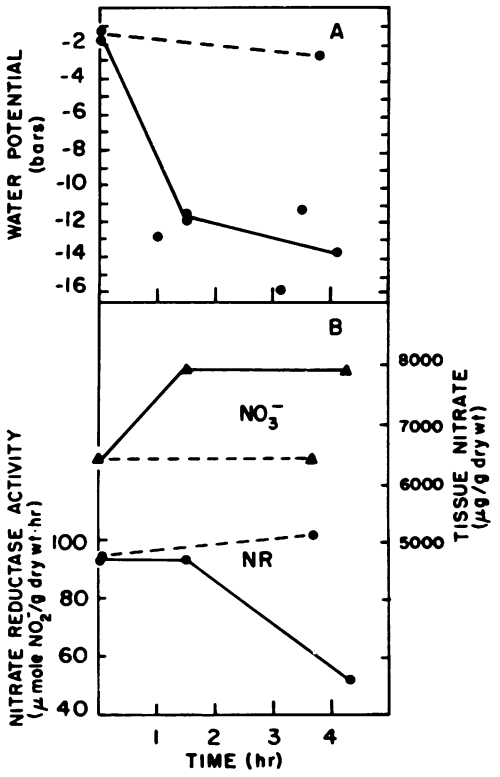

FIG. 5. Effect of 1.5 hr of desiccation on NR activity and tissue NO_3^- in excised shoots from 8-day-old seedlings. A: Changes in leaf water potential; B: changes in NR activity (●) and tissue NO_3^- content (▲). Desiccated tissue (——); controls, which consisted of excised shoots in water (– – –).

leaf Ψ_w decreased without a corresponding change in NR activity, there appeared to be no direct inactivation of the enzyme by the low leaf Ψ_w.

The nitrate content of the leaf samples increased by 20% during rapid desiccation, and measurements of the nitrate content of the entire excised shoots indicated that the increased concentration in the leaf was probably due to mobilization of nitrate from the stems to the leaves. This further confirms that the ultimate decline in NR activity was not due to low tissue nitrate, although the possibility again remains that the nitrate was compartmentalized and desiccation prevented translocation of the nitrate from inactive pools to the site of synthesis of the enzyme.

Synthesis of NR during Desiccation. Since inactivation of the enzyme by low Ψ_w did not account for the reduction in NR at low Ψ_w and neither the lack of induction of the enzyme by low tissue nitrate, nor the rate of decay of the enzyme appeared to be involved, low Ψ_w apparently affected NR activity mainly through a decrease in the rate of NR synthesis. The possibility of studying the effect of desiccation on the incorporation of leucine ^{14}C as a measure of protein synthesis was considered. However, since these studies have to be done in aqueous medium, it was necessary to determine first if osmotic solutions could cause decreases in NR activity proportional to decreases in Ψ_w. The effect of two osmotic solutions, 0.6 M mannitol ($\Psi_w = -15$ bars) and 0.5 M sucrose ($\Psi_w = -14.3$ bars) was compared with the effect of rapid desiccation in 8-day-old excised shoots. Enzyme activity in the presence of low levels of sucrose ($\Psi_w = -0.3$ bars) was used as a control. Although the shoots kept in the osmotic solutions looked wilted, the solutions did not reproduce the effect of desiccation on NR activity (Fig. 6) and therefore the use of leucine ^{14}C incorporation as a measure of protein synthesis was ruled out.

Another possibility was to investigate the distribution of

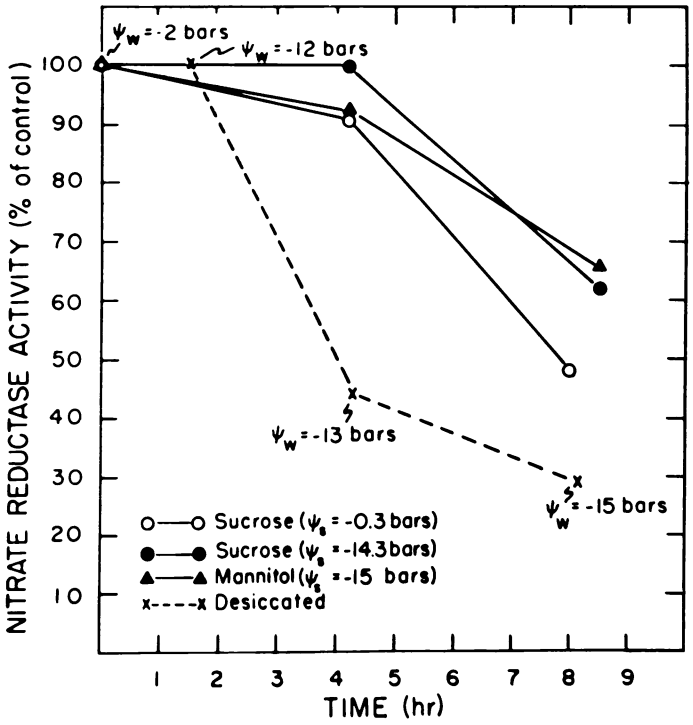

FIG. 6. Effect of osmotic solutions on NR activity in excised shoots from 8-day-old corn seedlings. Controls were intact, well watered seedlings.

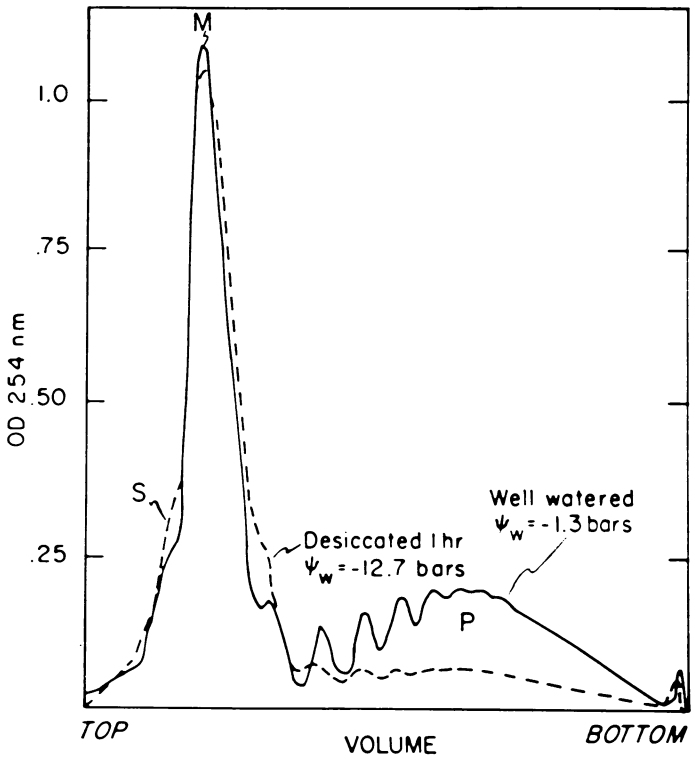

FIG. 7. Sucrose gradient profiles of ribosomes from well watered and desiccated 8-day-old, excised shoots.

ribosomes in the leaf as a function of Ψ_w. It has been shown that there is a good correlation between the polyribosomal content of leaves of corn seedlings and the rate of synthesis of NR (20, 21).

Figures 7 and 8A show that after 1 hr of desiccation (Ψ_w was −12.7 bars) there was a decrease in the polyribosomal content of excised corn leaves to 58% of the control. These results are consistent with the decrease in polyribosomal levels with decrease in Ψ_w previously reported (11). The decrease was not accompanied by a major increase in monoribosomes and subunits (Fig. 7), but instead was associated with a 10% decrease in the total ribosomal content of the tissue (Fig. 8A). After the initial changes in Ψ_w took place, the shoots were placed in a humid chamber, and no further major changes in Ψ_w, polyribosomes, or total ribosomes were observed (Fig. 8A). The changes in Ψ_w and NR activity in this experiment are similar to the data presented in Figure 5.

Since the decline in polyribosomal content preceded the reduction in NR activity at low Ψ_w (*cf.* Figs. 5, and 8A), the decrease in activity was consistent with a reduction in the rate of synthesis of the enzyme.

Changes in the polyribosomal content of the tissue occurred before changes in RNase activity at low Ψ_w (Fig. 8, A B). Thus, the reduction in polyribosomal content did not appear to be associated with changes in RNase activity. After 3 hr of desiccation, however, the activity of the RNase enzyme in the desiccated seedlings was 50% higher than in the control. Since no change in RNase activity was seen in the control during the 3-hr experiment, the increase in RNase during desiccation appeared to be due to the decrease in Ψ_w and not to excision. Similar increases in RNase activity have been reported as a result of inadequate water supply (7, 22). Tvorus (22) has indicated that the increase probably is due to *de novo* synthesis of the enzyme, since the response to desiccation could be prevented by cycloheximide. This suggests that certain pro-

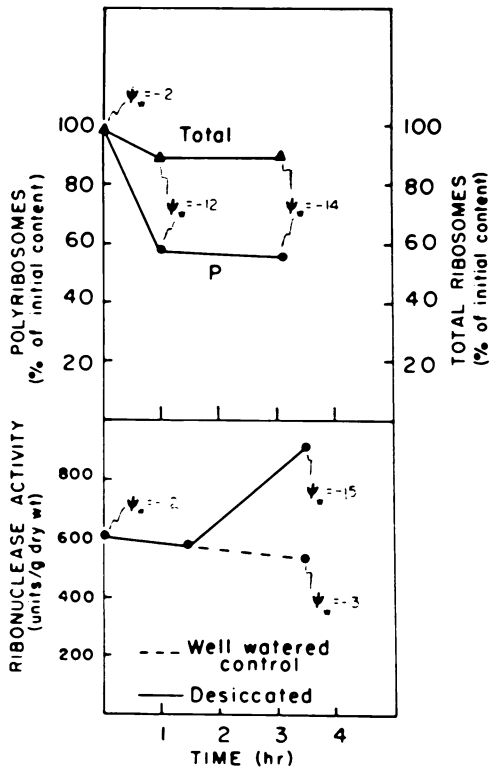

FIG. 8. Effect of 1 hr of desiccation on excised shoots from 8-day-old corn seedlings. A: Changes in total ribosomal content (▲) and percentage of polyribosomes (●); B: changes in RNase activity. The controls were excised shoots in water.

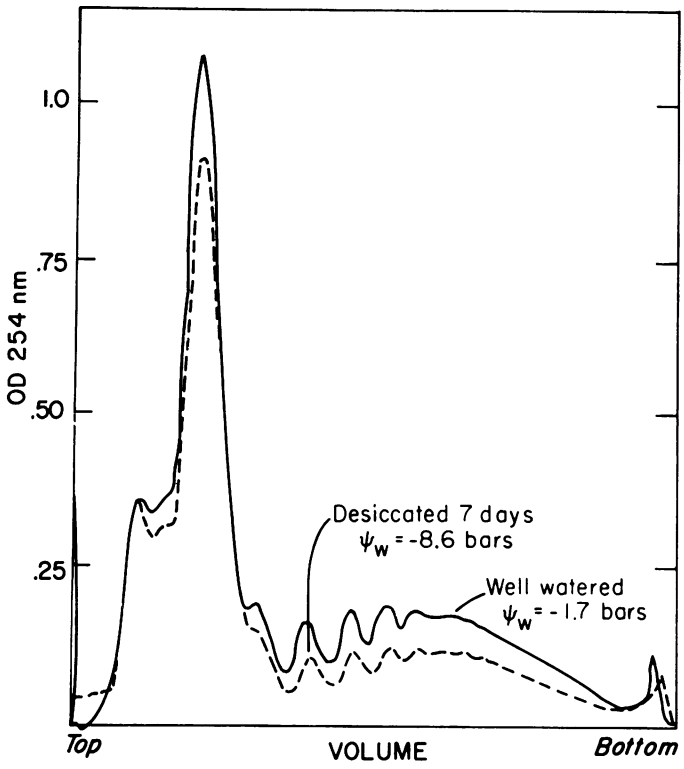

FIG. 9. Sucrose gradient profiles of ribosomes from well watered and desiccated 13-day-old, intact seedlings.

teins may be synthesized in desiccated tissue, despite the reduction in polyribosomal content of the tissue.

When intact plants were desiccated slowly over a period of several days, the polyribosomal and total ribosomal contents of the tissue also declined (Figs. 9 and 10A). The decrease in total ribosomes was associated with a decrease in monoribosomes as well as polyribosomes (Fig. 9). Ribonuclease activity was unchanged when desiccation was slight (Ψ_w of −3 bars) but almost doubled as desiccation became more severe (Fig. 10B). Thus, the changes in ribosomal profiles and RNase occurred during desiccation regardless of whether tissue had been excised and rapidly desiccated or remained intact and was desiccated slowly.

If protein synthesis is involved in the decrease in NR at low Ψ_w, NR activity should recover when plants are rewatered, and cycloheximide should prevent this recovery. This was verified by desiccating plants for 7 days prior to excision of the shoots. At the time of excision, the shoots had a Ψ_w of −7.6 bars and NR activity about 30% of the control. The cut ends of the shoots were then inserted into 20 mM phosphate buffer (pH 4.0) containing 50 mM nitrate, thus permitting rehydration. The Ψ_w of the desiccated shoots recovered in 20 min to the level of the controls and remained essentially constant for the remaining 6 hr (Fig. 11A). NR activity increased but more slowly than the increase in Ψ_w and continued to increase for 4 hr (Fig. 11A). When cycloheximide was present in the solution in which the shoots were recovering, no increase in NR activity was observed (Fig. 11A). Thus, the increase in activity of NR after recovery of Ψ_w apparently was due to *de novo* synthesis of the enzyme.

The polyribosomal content of the tissue also recovered following the recovery in Ψ_w (Fig. 11B). After 1 hr, the polyribosomal content had increased from an initial 32% to 44% of the total ribosomes. This recovery in polyribosomal content is consistent with the findings of Hsiao (11), who indicated that recovery from low Ψ_w caused the ribosomes to go back to the polymeric form. The polyribosomal content of the tissue declined again after the 3rd hr in the recovered seedlings, probably as a result of excision. A continuous decline in the total ribosomal content of the tissue also occurred both in the controls and the recovered seedlings during the 4 hr of the experiment (Fig. 11B, inset).

The concurrent recovery of percentage of polyribosomes and NR activity after 1 hr was reproducible and appeared related. The rate of recovery of polyribosomal content was more rapid than of NR activity and was complete after 1 hr. Hence, the data suggest that the increase in polyribosomal content preceded the increase in NR activity (Fig. 11, A and B). Also, the decline in NR after 4 hr of recovery followed, but at a slower rate, the decrease in polyribosomal content of the tissue (Fig. 11, A and B).

No correlation was found between the changes in the polyribosomal content of the tissue and the changes in RNase activity (Fig. 11C). RNase activity did not change for the first 3 hr after recovery was initiated, when most of the changes in ribosomal distribution occurred. After 3 hr, RNase activity increased at a similar rate both in the controls and in the desiccated seedlings, suggesting that this increase was due to excision.

These data show that low Ψ_w is associated with decreases in polyribosomes, total ribosomes, and NR activity. Rewatering of desiccated tissue resulted in an increase in polyribo-

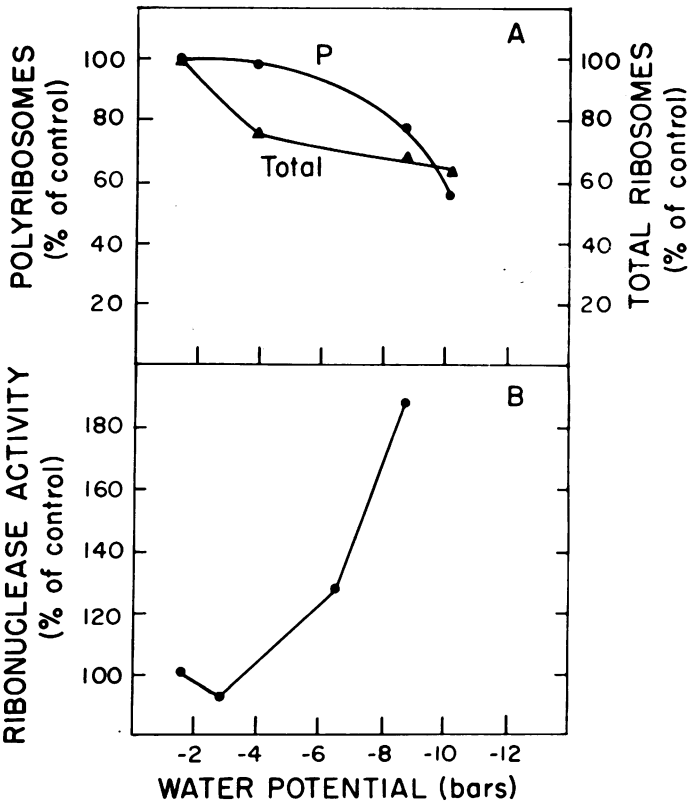

FIG. 10. Effects of decreasing water potential on RNase, polyribosomal and total ribosomal content of intact 8- to 13-day-old corn seedlings. Plant were desiccated by withholding nutrient solution from the root medium. Desiccation was started 6 days after planting. Controls remained well watered throughout the experiment. A: Changes in tissue content of total ribosomes (▲) and polyribosomes (●); B: changes in RNase activity.

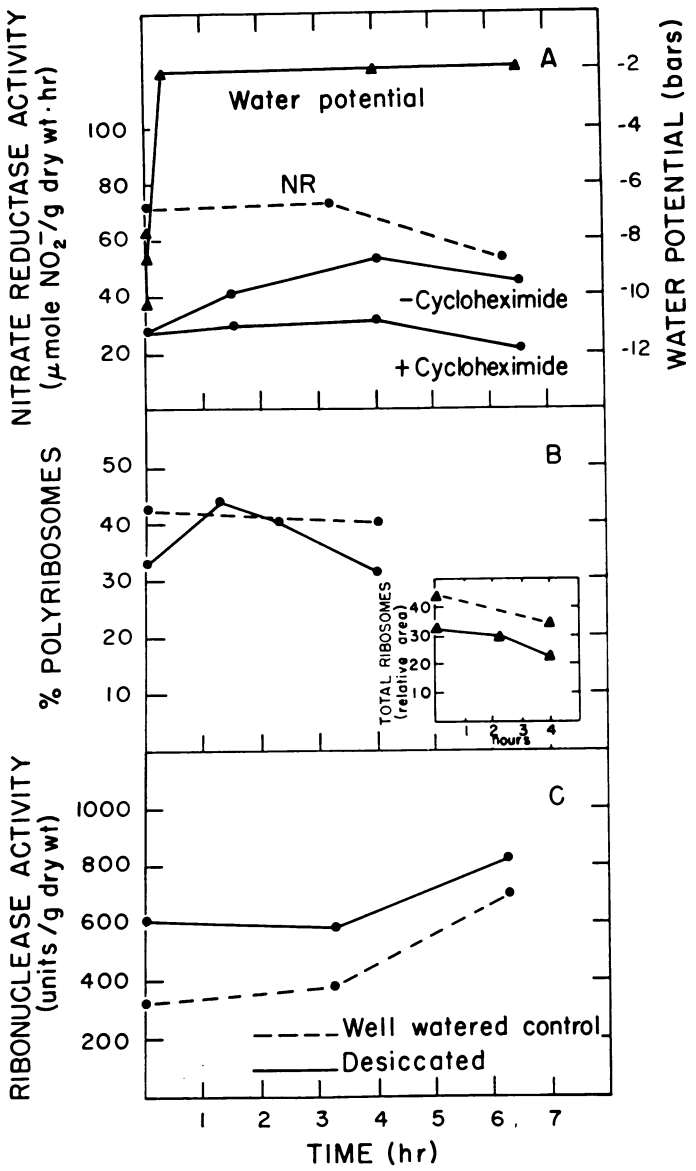

FIG. 11. A: Effect of cycloheximide on the recovery of NR activity in excised shoots from 13-day-old seedlings that had been desiccated for 8 days prior to recovery. The desiccated shoots were cut under degassed water and incubated in phosphate buffer (20 mM, pH 4) containing 50 mM NO_3^-, with and without 50 μg/ml cycloheximide. The controls were well watered, excised shoots that were treated identically but without cycloheximide. Changes in NR activity (●). Changes in leaf water potential (▲). B: Changes in total ribosomes (▲) and percentage of polyribosomes (●). C: Changes in RNase activity. Well watered control (– – –); recovered tissue (——).

somes and a subsequent increase in NR activity. This increase was prevented by addition of cycloheximide. Furthermore, decreases in NR activity at low Ψ_w were not associated with nitrate content of the tissue, increased degradation, or inactivation of the enzyme. Therefore, the decrease in NR at low Ψ_w appears to be due primarily to a decrease in the rate of enzyme synthesis.

While it is possible that low Ψ_w might have caused a direct inactivation of NR which was rapidly reversed during assay in an aqueous medium and consequently was undetected, this is not consistent with the slow activation of NR reported by Vennesland and Jetschmann (23). If such an effect occurred, it probably was small, since it has been shown that hydration of the enzyme urease results in only small changes in activity at hydration levels that are equivalent to those in cells at low Ψ_w (19). Thus, it appears unlikely that there were large direct but undetected effects of desiccation on NR.

The implication of protein synthesis as the source of changes in enzyme activity during desiccation suggests that changes in the activity of many enzymes should be largest in those with shortest half-lives, as proposed by Bardzik *et al.* (1). However, the data also suggest that the effects of desiccation cannot be considered to be simply a general reduction in the rates of all protein synthesis. RNase activity increased during desiccation and, according to Tvorus (22), involved *de novo* synthesis of the enzyme. Thus desiccation apparently resulted in a change in the kinds of enzymes that were synthesized as well as in a reduction in the overall rate of protein synthesis.

The increase in RNase during desiccation presumably could have reduced the levels of RNA susceptible to degradation in the tissue and possibly resulted in a general reduction in protein synthesis. This does not appear to have been the case in the present work. Increases in RNase activity always occurred after changes in ribosomal profiles were initiated (Figs. 8, A and B; 10, A and B; 11, B and C). Thus, unless a fraction of RNase which was undetected in our assay accounted for the changes in ribosomes, the increase in RNase appeared to be a consequence, rather than a cause, of at least the early ribosomal changes at low Ψ_w.

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