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Tissue Respiration and Mitochondrial Oxidative Phosphorylation of NaCl-Treated Pea Seedlings[1]

Avinoam Livne and Naomi Levin

Department of Plant Physiology, The Negev Institute for Arid Zone Research, Beersheva, Israel

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Summary. The effect of sodium chloride added to root medium of pea seedlings on respiratory activity of tissue segments and on isolated mitochondria was studied. Salinization enhances the respiration of leaves about one-third on a fresh weight, dry weight or protein basis. Roots and stems show only 10 to 15 % respiratory stimulation. The onset of respiratory increase in leaves roughly parallels the increase in NaCl content and the decrease in growth rate. At a later stage the elevated respiration is apparent in treated plants even though the concentration of NaCl reaches a plateau and osmotic adjustment is being reached. Stimulation of respiration was found in both etiolated and green plants. Experiments with DNP show that simple uncoupling by salt is not involved; the respiratory increase in control and treated tissue is proportionally the same.

In accordance with increased respiration rates observed in vivo, mitochondria from salt-treated plants show higher rates of oxygen uptake on several substrates. The effect of NaCl added during growth is long term and is distinct from the effect of NaCl added to mitochondria isolated from control plants. Since P/O ratios are not affected by NaCl, the potential for oxidative phosphorylation in salt-affected tissue appears to increase. It is postulated that this increase may lead to changes in ADP and ATP content, and in turn, affect regulation of metabolic pathways.

Depression of plant growth by excess salts in the root medium is a well-known phenomenon (5, 8, 18). Plants differ greatly in tolerance toward saline media, but the physiological basis for the difference between tolerant and sensitive plants is not known. In order to understand the nature of growth depression by salts a detailed study of the metabolic responses of salt-sensitive plants appears of value.

Nieman (18) examined 12 crop plants and reported that leaves of salt-treated plants, particularly of salt-sensitive species, showed increased respiration rates; the response of roots was inconclusive but pea roots grown in saline medium showed elevated respiration. Porath and Poljakoff-Mayber (19) reported, however, that NaCl increments reduced respiration of pea root tips. In view of these conflicting reports, a reexamination of the problem seemed desirable.

This communication reports on the effects of NaCl increments on tissue respiration during plant growth. In order to examine the involvement of mitochondria in the response of the tissue to salt treatment, the oxidative and phosphorylative activities of mitochondria isolated from salt-treated and control plants was studied.

Peas were chosen for this study because of their growth sensitivity to sodium chloride (5, 18) and in view of the extensive study of pea mitochondria by Smillie (21, 22, 23). An abstract of part of this study has appeared (14).

Materials and Methods

Plants were grown in a cooled greenhouse throughout the year. Temperature at the height of the plants varied—day maxima of 21 ± 3°, night minima of 15 ± 2°. Laxton Progress peas were germinated in moistened vermiculite and 3 day-old seedlings then transferred to nutrient solution in 2.5 liter, light-proof, plastic containers. The roots of about 60 seedlings were inserted in each container through holes in the cover. The half-strength Hoagland nutrient solution contained, in mM: $Ca(NO_3)_2$, 2.5; KNO_3, 2.5; $MgSO_4$, 1; KH_2PO_4, 0.5; and in mg/l: Sequestrene-iron (Fe EDTA), 25; H_3BO_4, 1.45; $MnCl_2$, 0.9; $ZnCl_2$, 0.06; $CuCl_2$, 0.024; with pH adjusted to 5.5 with KOH

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or KH_2PO_4. The solution was continuously aerated and changed every 2 to 3 days.

The osmotic potential (OP) of the base nutrient solution was about 0.4 atm and was considered as zero (control) salinity. Salinization was brought about gradually by adding 24 mM NaCl (or more, according to activity coefficient) each day for 3 days producing a final concentration of 77 mM and a final OP of 3 atm.

In some experiments (table III), immediately after NaCl increment, control and treated plants were grown at 20°, either under continuous light (Sylvania GroLux Lamps, 7500 lux at the height of the plants) or in the dark.

Oxygen uptake was measured manometrically in triplicates at 30° using 100 mg (fr wt) of tissue segments. About 100 mg fresh weight of leaf and stem tissue were placed in a vessel containing 2 ml of 0.1 M potassium phosphate buffer (pH 6.0), while for root tissue 2 ml of nutrient medium (salinized for treated tissue) were used. Leaves were cut into about 0.6 × 0.6 cm sections with a razor blade; approximately 1 cm segments were used for stems and roots. Root segments were cut from the central part of 8 to 12 cm-long secondary roots. In the 1 to 3 days after all NaCl increments the second leaf was analyzed, while the third leaf was used thereafter (except for table III).

For preparation of mitochondria, samples (40 g fr wt of plant tops) were chilled (2–4°, 15 mins) and homogenized with 80 ml of preparation medium (0.4 M sucrose, 5 mM EDTA-tris, 40 mM potassium phosphate, pH 7.4) with sodium chloride added as noted in Results. Samples were ground as uniformly as possible in a mortar, or homogenized in a Waring Blender at half speed for 3 5-second runs, so that intact tissue could be pushed back into the brei. The brei was passed through muslin, centrifuged at 1000 × *g* for 5 minutes and the supernatant material spun at 9000 × *g* for 10 minutes. The sediment of the high speed centrifugation was washed by resuspension in the preparation medium and centrifuged at 9000 × *g* for 10 minutes. Occasionally, a second washing was made. The washed pellet was suspended in 1 to 2 ml of preparation medium and aliquots of 0.2 ml representing 2 to 4 g fresh weight and containing 6 to 15 mg protein were used for measurements of oxidative phosphorylation. Chlorophyll was present in the mitochondrial fraction at 10 to 25 μg per mg protein.

Measurements of oxidative phosphorylation were carried out either manometrically in a Warburg apparatus at 30° or polarographically at 26°. Phosphorylation was determined in the manometric studies by measuring glucose-6-P according to Heytler (9).

For dry weight determination, samples were dried at 80° under reduced pressure for 24 hours. Chloride ions were extracted from the dried samples with 0.1 N HNO_3 and determined by a Buchler-Cotlove Chloridometer. Sodium and potassium were analyzed by flame photometry in samples digested with HNO_3. Osmotic potential of leaf sap was determined cryoscopically using a calibrated thermistor. For protein analysis, samples were homogenized with 5% trichloroacetic acid, the precipitate washed with the same reagent, then with acetone, and finally suspended in 50 mM NaOH. Protein was determined colorimetrically with the Folin-Ciocalteu reagent (15). Nitrogen was determined by the Nessler reagent.

Fatty acid poor bovine albumin (Lot 62736) was obtained from Calbiochem. Carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) was kindly supplied by Dr. P. G. Heytler of E. I. DuPont de Nemours & Co. All other chemicals were purchased from Sigma Chemical Co.

Results and Discussion

Plant Growth. In line with previous reports (5, 18), NaCl added to the root medium of pea seedlings reduced weight and size of the plants. The effect was greatest in leaves and smallest in roots. Figure 1A traces the fresh weight of a shoot affected by NaCl increments. When these data were expressed on dry weight basis, the differences between control and salt-treated plants appeared smaller by 5 to 10%, but were still significant. The rate of appearance of new leaves was not affected by salt. In contrast to Nieman's report (18), treated plants completed their life cycle even on relatively highly saline media probably because of more favorable growth conditions. Media of up to 120 mM NaCl were tested for that purpose.

Osmotic Adjustment and Ion Uptake. The OP values of leaf sap given in figure 1B show that within 1 week of NaCl additions treated plants approached an osmotic adjustment (3, 4). Apparently, the adjustment was achieved, at least partly, by uptake of sodium and chloride ions as shown in figure 1C. A plateau of the contents of these ions in leaves of treated plants was apparent when their concentration was similar to that in the external medium. The level of potassium ions is considerably lower in leaves of treated plants than in the control ones, probably because of ion competition.

Respiration. On fresh weight basis, leaves of treated plants had consistently higher respiratory rates than control ones, figure 1 showing that these prevailed both during the phase of uptake of sodium and chloride ions and after it, when the level of these ions reached a plateau. Results were similar when rates were expressed per unit area or (see table II) per dry weight or protein unit. The NaCl-induced elevation of respiration was greatest in leaves and smallest in roots (table I), like the depression of growth. This relationship correlates with the observation (18) that respiration

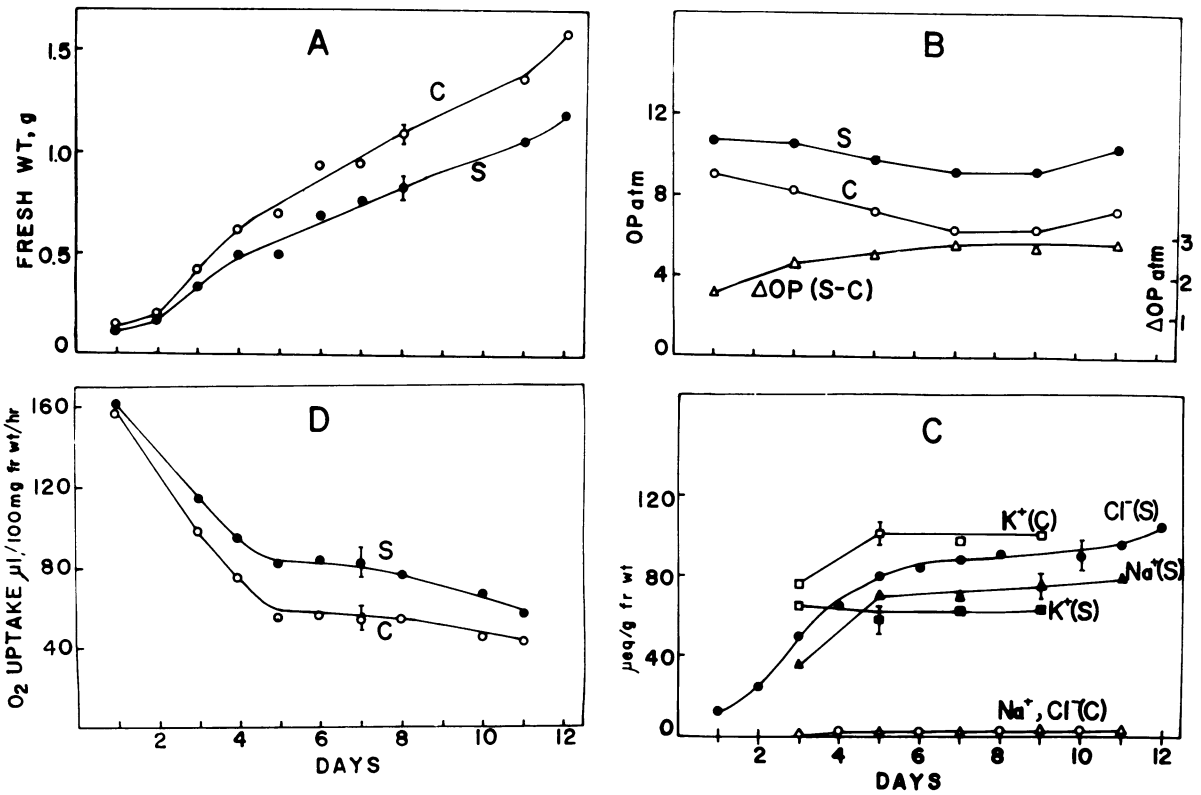

FIG. 1. Plant growth, ion content and respiration of pea seedlings as affected by NaCl. Abscissae denote days after increment of 77 mM NaCl. "C" is control plant and "S" salt-treated plant. The values are an average of 3 experiments and representative standard errors given in vertical lines. A) growth of the plants as g fr wt per shoot. B) OP of leaf sap. C) contents of Na^+, K^+, and Cl^- in leaves as μeq per g fr wt. D) respiratory rates of leaf sections.

Table I. *Effect of NaCl on Respiratory Rates of Parts of 13 Day-Old Pea Seedlings*
Treated plants grown for 6 days in 77 mM NaCl.

Plant part	Medium	Oxygen uptake		% Control
		Control	Salt-treated	
		μl per 100 mg fresh wt per hr		
Leaves	Buffer	65.1	90.3	+38
Stems	Buffer	23.2	26.7	+16
Roots	Nutrient solution	27.0	29.8	+10
Root tips	Nutrient solution	48.2	54.2	+12
Root tips	Nutrient solution + 1.8 mM glucose	54.4	62.5	+11

Table II. *Effect of NaCl and Plant Size on Respiratory Activity of Leaves of 15 Day-Old Pea Seedlings*
Treated plants were grown for 7 days in 77 mM NaCl.

	Control plant		Salt-treated plant	
	Large	Small	Large	Small
Shoot fr wt, g	1.09	0.68	0.75	0.43
Leaflet area index*, cm^2	6.23	4.01	4.16	2.38
O_2 Uptake, μl/hr				
Per 100 mg fr wt	33.7	39.3	48.3	52.4
Per 10 mg dry wt	37.3	41.3	50.0	51.0
Per mg protein	19.8	21.3	26.3	26.2

* Length times width.

was more greatly stimulated in species whose growth was more greatly depressed by NaCl.

The respiratory rates of root sections declined following several hours of incubation in aerated medium. The decline was more pronounced in treated than in control tissue. This may explain in part the lower respiratory rates of salt-grown roots reported by Porath and Poljakoff-Mayber (19), since they incubated the sections for several hours prior to measurement of O_2 uptake.

Higher respiratory rates in the salt-treated plant may result from reduced growth rather than from a direct salt effect. Table II describes an experiment designed to check this possibility. Large and small plants were chosen from both control and salt treatments. Plants in each group were judged large or small according to shoot weight and leaf area, these 2 parameters having very good correlation. Table II shows that smaller plants do indeed have higher respiratory rates, particularly on fresh weight basis. However, the difference in respiration between control and treated plants is well apparent, regardless of plant size or the basis for expression of respiratory rates. It is, therefore, concluded that elevated respiration is not merely a consequence of reduced plant growth.

Decrease in photosynthetic activity (18) and an apparent increased participation of the pentose phosphate cycle (19) in salt-treated peas raised the question of a possible analogy of salt effects to the effects of obligate parasites on the plants. In the case of rust (13) and powdery mildew (20) it has been suggested that elevated respiration and participation of the pentose phosphate cycle in infected tissue are causally associated with decline of photosynthesis. Scott and Smillie (20) reported that etiolated plants infected with the mildew fungus do not show elevated respiration as do green infected plants. Table III shows that leaves of salt-treated plants have higher respiratory rates no matter the potential for photosynthetic activity. The analogy of our system to an infected plant is further weakened by Nieman's observation that, in contrast to peas, photosynthesis was not suppressed by NaCl in many other plant species, while respiration was stimulated.

Accumulation of salt and maintenance of a higher OP presumably result in greater energy requirement and expenditure in the salt-treated plant. A higher ATP turnover may thus be envisaged. In view of the role of the adenylate system in controlling respiratory rates in plant tissues (2), it is tempting to suggest that the higher rates of respiration in salt-treated plants are caused by greater availability of P_i and ADP. If this were the case, it could be expected that tissues of control and salt-treated plants would show equal rates of respiration when treated with an uncoupler, like DNP, at the most effective concentration. Figure 2 shows that the maximum respiratory stimulation induced by DNP is actually greater in leaves of salt-treated seedlings than in control. A similar ratio between respiratory rates in salt-affected and control tissues was obtained without DNP so that an increased turnover of the adenylate system is not the reason for the increased respiration caused by NaCl.

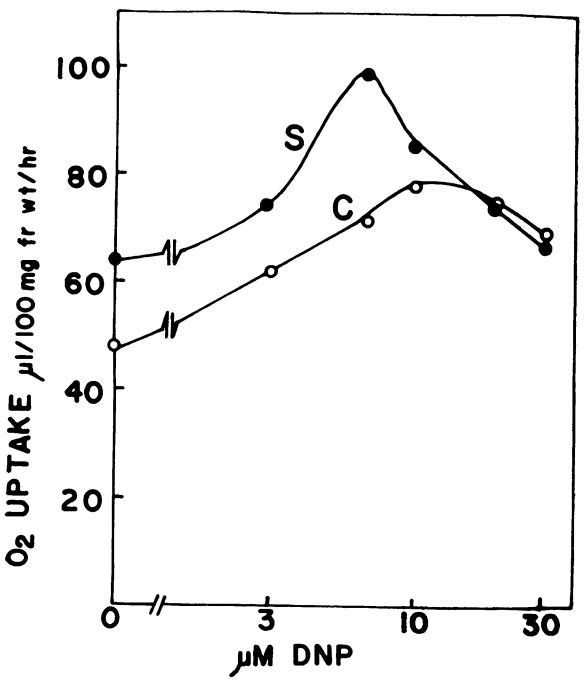

FIG. 2. Effect of DNP on respiration of leaf sections from 13 day-old control and salt-treated pea seedlings. Treated plants were grown in 77 mM NaCl for 5 days. Respiration was measured in pH 5.0 phosphate buffer, rates computed from the first hour.

Table III. *Effect of NaCl on Respiration of Green and Etiolated Pea Plants*

After increment of NaCl (77 mM), control and salt-treated plants were grown for 14 days at 20°, either under continuous light or in the dark. The data are oxygen uptake by sections of the fifth leaf.

Plant	Oxygen uptake		
	Control	Salt-treated	% Control
	µl per 100 mg fresh wt per hr		
Green	92.2	128 8	+40
Etiolated	71.6	97.1	+35

Table IV. *Effect of NaCl in Growth Medium on Oxidative Phosphorylation of Pea Seedling Mitochondria – Manometric*

The reaction mixture consisted of 0.4 M sucrose, 20 mM glucose, 10 mM $MgSO_4$, 14 mM K_2HPO_4-KH_2PO_4 (pH 7.4), 2.5 mM ADP, 20 mM succinate, 2 mg/ml albumin, 50 μM CoA, 0.2 mM TPP, 0.2 mM DPN and 50 K-Units/ml hexokinase in a total volume of 2 ml.

Plants*	NaCl (80 mM) during mitochondrial preparation & analysis	O_2 Uptake (μatoms/hr per mg N)	P_i Esterified (μmoles/hr per mg N)	P/O
Control	—	6.12	11.28	1.84
	+	4.92	9.00	1.83
Salt-treated	—	11.40	21.84	1.92
	+	9.78	20.16	2.06

* Fifteen day-old plants; salt-treated plants grown for 8 days in 77 mM NaCl.

Table V. *The Effect of Several Additives on Respiratory Control Ratio and Oxidative Phosphorylation of Pea Seedling Mitochondria*

The basic reaction mixture for the polarographic measurement consisted of 0.4 M sucrose, 5 mM $MgCl_2$, 17 mM tris HCl (pH 7.4), 5 mM K_2HPO_4 – KH_2PO_4 (pH 7.4), 2 mM sodium succinate, 4 mM DL-potassium glutamate; ADP added at increments of 0.1 mM. The additives examined were 10 mM $MgCl_2$, BSA as specified and the cofactors: 40 μM CoA, 0.1 mM TPP and 0.2 mM DPN.

Additives to reaction mixture	Respiratory control ratio	Q_{O_2} (State 3) μatoms O_2 uptake/hr per mg protein	ADP/O
None	1.5	1.08	1.42
Cofactors, Mg^{++}, BSA (1 mg/ml)	2.2	1.15	1.50
Cofactors	1.5	0.80	1.55
Mg^{++}	1.6	0.96	1.53
BSA (1 mg/ml)	2.2	1.25	1.40
BSA (3 mg/ml)	2.7	1.17	1.52

Table VI. *Effect of NaCl on Oxidative Phosphorylation; NaCl Added During Growth of Pea Seedlings, Preparation of Mitochondria or Analysis – Polarographic*

The complete reaction mixture contained: 0.4 M sucrose, 5 mM K_2HPO_4 – KH_2PO_4 (pH 7.4), BSA 2.3 mg/ml, 3.75 mM sodium pyruvate, 7.5 mM DL-potassium malate, ADP added at increments of 0.25 mM; NaCl (when specified) was 75 mM.

Plants*	Preparation medium**	Reaction mixture	Respiratory control ratio	Q_{O_2} (State 3) μatoms O_2 uptake/hr per mg protein	ADP/O
Control	SEP	Complete	4.1	2.2	1.4
		+NaCl	4.2	1.8	1.6
		—BSA	2.3	1.7	1.7
Salt-treated	SEP	Complete	4.8	3.2	1.5
		+NaCl	6.1	2.8	1.7
		—BSA	3.7	3.1	1.4
Control	SEP + NaCl	Complete	2.6	1.4	1.3
		+NaCl	2.8	1.7	1.3
		—BSA	1.8	1.2	1.7
Salt-treated	SEP + NaCl	Complete	3.4	2.7	1.5
		+NaCl	4.1	2.9	1.7
		—BSA	3.2	2.3	1.5

* Thirteen day-old plants; salt-treated plants grown for 6 days in 77 mM NaCl.

** SEP denotes sucrose-EDTA-phosphate medium. Sodium chloride added at 75 mM.

Both tissues responded to DNP without a lag but the optimal concentration of DNP for salt-treated tissue was lower, possibly due to faster uptake of the uncoupler. The uncoupler *m*-Cl-CCP (9) was tested at 0.1 to 50 μM at pH range of 6.0 to 8.5 but showed no clear responses, possibly because of its low solubility and slow penetration into intact plant cells.

The pattern of response of the tissues to DNP suggests that the elevated respiration induced by NaCl is mediated by an effect on mitochondria, as already proposed by Nieman (18). Experiments described below, with isolated mitochondria appear to substantiate this supposition.

Oxidative Phosphorylation. A typical, manometrically-analyzed experiment with succinate as substrate (table IV) shows that mitochondria from salt-treated plants have a higher rate of oxygen uptake, while P/O ratios are not appreciably affected. Essentially the same was found when α-ketoglutarate was used as substrate.

The greater respiratory activity might have been caused by exposure of the mitochondria of treated plants to the higher electrolyte content released by homogenization. To check this possibility, NaCl was added to the preparation and reaction media at a concentration equivalent to the chloride ions present in the treated tissue. This addition of NaCl actually reduced oxygen uptake in mitochondria of both treated and control plants (table IV). Therefore, the respiratory rates observed for mitochondria from treated plants are probably minimal.

The cofactors listed in table IV were included because Smillie reported (21) that pea mitochondria require undefined coenzyme concentrate from yeast. As can be seen from table V, these cofactors had no effect in our system; nor did cytochrome C. Thus, these were all omitted in subsequent experiments.

When the preparation procedure described in Methods was employed, polarographic measurements revealed mitochondria with respiratory control ratios (6) commonly of 2 to 2.5, with occasionally higher values. Lowering the pH of the media, the use of a high mannitol concentration, omission of Mg^{++}, as well as other modifications concerning EDTA and cysteine for improved mitochondrial preparation, as recommended by Verleur (24), were confirmed by us for potato tuber tissue. However, these modifications failed to improve the respiratory control ratio of pea seedling mitochondria. The modified centrifugation sequence suggested by Lyons, et al. (16) was also ineffective. As reported by Lance, et al. (12) and Verleur (24), the presence of albumin, particularly in the reaction mixture, was critical for respiratory control (tables V and VI). Crystallized bovine serum albumin (BSA) and fatty acid poor albumin gave identical results. Interestingly, the respiratory control ratios of various treatments within a single experiment appeared to be reproducibly scaled, despite variation within experiments. Mitochondria from treated plants exhibited higher respiratory control ratios than those from normal plants (table VI). This was apparent also when aliquots of equal protein content were analyzed. Furthermore, mitochondria from normal plants were relatively more affected by the presence of albumin in the reaction mixture. Addition of 75 mM NaCl to the preparation medium was deleterious to the respiratory control ratios of mitochondria from both treated and control plants. However, when added to the reaction mixture, NaCl did not decrease these ratios.

Polarographic measurements given in table VI for pyruvate and malate further show that mitochondria from salt-treated plants have higher

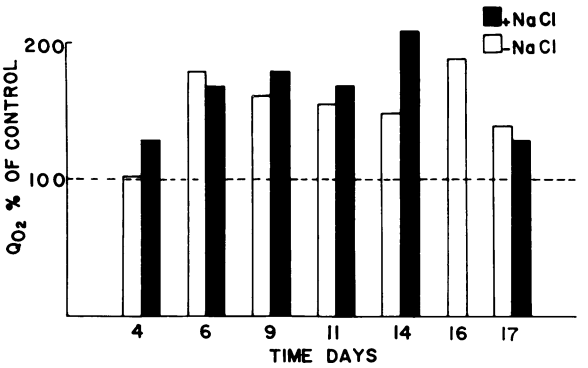

FIG. 3. Oxygen uptake by mitochondria from salt-treated pea seedlings as a function of time of plant growth in 77 mM NaCl. Values for mitochondria from comparable control plants are expressed as 100 %. Open bars) reaction mixture without NaCl; Solid bars) NaCl (75 mM) added to reaction mixture. The procedure and composition of reaction mixture as in table III.

Table VII. *Content of Sodium and Chloride Ions in Mitochondria from Control and NaCl-Treated Plants*

Mitochondrial preparation*	Ion	Control plants	Salt-treated plants
		μeq/mg protein	
Washed once	Na+	1.3	4.8
	C—	1.4	3.3
Washed twice	Na+	0.8	1.0
	C—	nil	nil

* See Materials and Methods.

respiratory rates than the controls. This was also true for other substrates used: succinate, α-ketoglutarate, or succinate-glutamate. The differences in respiratory activity of the mitochondria or salt-treated and control plants persisted no matter the buffer (tris or phosphate) or the sucrose molarity (0.15–0.5) used. Sodium chloride had very little effect on ADP/O ratios whether applied during growth of the plant, during preparation of mitochondria, or to the reaction mixture. Significantly, the higher oxidative activity of mitochondria from salt-treated plants is in accordance with the elevated respiratory rates of tissue sections from salt-treated plants. There are 2 lines of evidence establishing that this greater mitochondrial activity is not merely due to the higher electrolyte content in the extracts of salt-treated plants: A) the stimulatory effect of NaCl added during the growth period could not be duplicated by NaCl added during the preparation of mitochondria from control plants; B) twice-washed mitochondria from both control and treated plants differed only slightly in content of sodium and chloride ions (table VII), while their difference in respiratory rates prevailed. These considerations call for 2 types of effect of NaCl on plant mitochondria: A) an immediate effect, as has been reported for ion-dependent stimulation of oxidative activities of mitochondria (7, 11, 17), and B) a long-term effect, as mentioned by Porath and Poljakoff-Mayber (19) that, despite qualitative differences in results, also applies for the system described here.

Figure 3 demonstrates that the increase in respiratory activity of mitochondria from salt-treated plants, as compared to controls, is long-lasting, but that it is subjected to some variation with age. Since variations of similar magnitude were common in day-to-day preparation, no particular tendency in the response of the treated tissue can be seen.

The involvement of adenosine phosphates (ATP, ADP, AMP) in many reactions, either as substrates or as effectors in the regulation of metabolic pathways, is well known (1). The present study shows that oxidative phosphorylation is higher in salt-treated than in control tissues. Thus, there may be an alteration in the steady state concentration and in the relative proportions of the adenosine phosphates in salt-treated plants, leading in turn to an upset of normal metabolic pathways. Work in progress (Livne, unpublished) shows that saline medium does indeed substantially affect the ratio of ATP/ADP in pea seedlings.

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