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Characterization of the Mobile Charges in the Membrane of *Valonia utricularis*

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Summary. An attempt for the characterization of the mobile charges has been made by investigation of the voltage relaxations following a charge pulse at various temperatures between 2 and 40°C. The translocation rate k, the membrane conductivity $1/R_m$ and the total concentration of mobile charges N_t within the membrane were calculated from recently developed theory (U. Zimmermann, K.-H. Büchner and R. Benz, *J. Membrane Biol.* **67**:183–197, 1982). Data from 21 cells show that the concentration of mobile charges does not change significantly over a temperature range of 5 to 34°C, whereas both the translocation rate and the membrane conductivity reveal a strong but reversible temperature dependence. In the temperature range of 18 to 5°C, k decreases by a factor of 10 to 20, while between 18 and 34°C the increase in k is only two- or threefold with a maximum around 25°C. In principle, the same temperature dependence was observed for the membrane conductivity. Hysteresis effects occurring in the low temperature range as well as at high temperatures indicate that a protein component is involved in the mobile charge system. Furthermore, addition of cycloheximide leads to a marked decrease in both the translocation rate and the membrane conductivity, however, leaving the concentration of mobile charges almost unchanged. Therefore, it is assumed that the mobile charges are coupled to, but not part of a carrier protein.

Key Words charge-pulse technique · mobile charges · temperature dependence · hysteresis effects · cycloheximide · *Valonia utricularis*

Introduction

Charge-pulse experiments recently performed on cells of the giant alga *Valonia utricularis* strongly support the view that there are mobile charges in the membranes of this alga [28]. These mobile charges carry a negative charge and apparently play an important role in the sensing of turgor pressure changes. Increases in turgor pressure lead to a significant rise in the translocation rate of these mobile charges; this rise can be explained in terms of an electromechanical compression of the membrane [26, 28]. The change in the translocation rate of the mobile charges is observed in the pressure range of 0 to 2 bar. In this pressure range the potassium influx and efflux are pressure dependent [26, 29]. This correlation could indicate that transport processes in the membrane are directly regulated and controlled by pressure via mobile charges in combination with electromechanical compression of the membrane.

The nature of these mobile charges is still unknown. For example, they could be compounds of low molecular weight involved in metabolism, or hormones, or even proteins as part of a carrier system (e.g. of potassium or protons).

As charge movements have also been observed in neurons, indicating dislocation of gating particles [1, 4] and in impedance measurement on the giant alga *Acetabularia* [17, 23] one can readily assume that the appearance of mobile charges is a widespread membrane phenomenon. In this communication we have investigated on cells of *V. utricularis* the temperature dependence of the translocation rate, the concentration of the mobile charges and the membrane resistance. All three parameters can be determined from charge-pulse experiments if the latter are based on the appropriate model concepts. As will be shown in the following, the temperature dependence of these three parameters indicates that the mobile charges are most probably connected to proteins. Measurements of the voltage relaxation in the presence of cycloheximide, an inhibitor of protein synthesis, support this view. Under these conditions, the translocation rate is seen to decrease dramatically.

Materials and Methods

Cells of *Valonia utricularis*, originally obtained from the Mediterranean Sea near Naples, Italy, were grown in natural seawater at a salinity equivalent to 1130 mOsm/kg under continuous light as described earlier [29]. Geometrically even cells with volumes

between 26 and 190 μl were employed for investigation with the charge-pulse technique. The cells were fixed in a small Perspex® chamber perfused with seawater of constant temperature. During the experiments the temperature was varied in the range of 2 to 40°C and carefully monitored by a thermistor mounted very close to the cell. Unless stated otherwise, pH of the seawater was 8.2.

The procedure of the charge-pulse technique has been described in several publications in detail [5, 6, 7, 9, 24]. Briefly, membranes of the algal cells are charged up through an internal electrode to 5 to 20 mV by a very short square pulse of 200 to 1000 nsec duration. Right after the pulse, the current circuit is switched to a high impedance so that the subsequent membrane discharge monitored by a second pair of electrodes must be due to intrinsic membrane properties. Membrane discharge was recorded on a Nicolet digital storage oscilloscope Explorer III and evaluated by a Digital Equipment 1.1 MINC Lab Computer, whereas the charge pulse, i.e. the amount of charge used to polarize the membranes, was recorded on an HP storage oscilloscope 7633 and evaluated from Polaroid screen photography by planimetry. For inhibition of protein synthesis, solutions of 0.1 to 3 mg/ml of chloramphenicol and cycloheximide in seawater were used, both available from Serva/FRG.

Theory

The assumed transport system is based on the same model as proton transport mediated by uncouplers of the linear type [6, 8] and has been extensively described in several publications [11, 28]. The phenomenological description is basically the same as that of gating particles in neurons [1, 4]. The translocation of the neutral carrier-substrate complex is treated as a first-order reaction with a voltage-independent speed constant. The translocation rate of the charged carrier is assumed to be voltage dependent. The substrate concentration must be small as compared to the carrier concentration and the membranes must separate almost equal substrate concentration. Given these preconditions the model allows the calculation of the translocation rate k of the charged carrier at $\Delta U = 0$ as well as the surface density N_t of the charges and the electrical resistance of the membranes R_m from the experimentally obtained relative amplitudes a_1 and a_2 and time constants τ_1 and τ_2, respectively, of the voltage relaxations.

$$k = \frac{a_1 \cdot \tau_1 + a_2 \cdot \tau_2}{2 \cdot \tau_1 \cdot \tau_2} \tag{1}$$

$$R_m = \frac{a_1 \cdot \tau_1 + a_2 \cdot \tau_2}{C_m} \tag{2}$$

$$N_t = \frac{4 \cdot R \cdot T \cdot C_m}{F^2} \cdot \frac{a_1 \cdot a_2 \cdot (\tau_1 - \tau_2)^2}{(R_m \cdot C_m)^2} \tag{3}$$

where C_m = membrane capacitance, F = Faraday constant, R = gas constant and T = absolute temperature. Equations (1–3) correspond to Eqs. (19, 22) in Ref. [28] after rearrangement and substitution. Activation energies E_A were calculated from Arrhenius plots according to

$$\ln y = -\frac{E_A}{R \cdot T} + A \tag{4}$$

where y is the parameter under investigation (either $1/R_m$, K or N_t) and A is the intercept on the ordinate.

Results

The temperature range in which cells of *Valonia utricularis* can be maintained for longer periods of time without irreversible changes in the membrane structure, membrane transport and cellular functions is relatively narrow [14]. The range covers about 15 to 25°C and corresponds approximately to temperatures that might occur in the cells' natural environment. *Valonia* cells can be exposed to temperatures distinctly above or below this range for only short periods of time (10 to 30 min) without deterioration of the cells. For this reason, charge-pulse experiments were first performed at 18°C; the temperature was then either lowered (say to 6°C) or raised (say to 30°C) and voltage relaxation measurements were carried out after 10 min. The temperature was then returned to 18°C for about 30 to 60 min and measurements were repeated at this temperature, before subjecting the cells to a further temperature change. Voltage relaxations were also recorded at various temperatures while the temperature was being reduced to lower values or increased to higher values. This procedure enabled us to obtain a clear picture of the reversibility of the observed changes in the voltage relaxations. Only those experiments in which the original time constants of the voltage relaxations could be reproduced at 18°C were evaluated. Experiments performed on 21 cells demonstrated that under these conditions the changes in voltage drop at the two membranes are reversible if the temperatures were set between about 5 and 34°C. As a rule, two voltage relaxations are observed in this temperature range, their time constants differing by a factor of about 10. In some cases a third relaxation was observed with a time constant between the fast and

the slow one. This so-called intermediate relaxation had previously been observed. Since the amplitude of the intermediate voltage relaxation represents only a fraction of the total voltage amplitude it was ignored in the evaluation (for a detailed discussion, *see* [28]).

The time constants of the fast and the slow relaxations are strongly temperature dependent. At 18°C the time constant of the fast relaxation is about 100 μsec, while at 6°C it assumes values of 1 to 3 msec. At higher temperatures (30°C and above) the change is less pronounced. At a temperature of 34°C the time constant is about 300 to 600 μsec. Correspondingly, a marked increase in the time constant of the slow component of the voltage relaxation is observed at low temperatures (*see* Table 1), while towards higher temperatures the increase is less marked.

Figure 1 shows the semilogarithmic plots of typical voltage relaxations recorded from the same cell at 5, 18 and 34°C. In all cases the relaxations consisted of two exponential decays, so that each semilogarithmic plot yielded two straight lines from which the time constants could be determined. Using Eqs. (1, 2, 3), the translocation rate and the concentration of the mobile charges as well as the membrane resistance could be calculated. Figure 2 shows the dependence of the translocation rate k on the reciprocal value of the absolute temperature for the same cell as in Fig. 1. The direction of the arrow indicates the direction of the temperature change. The translocation rate decreases continuously from 480 sec^{-1} at 18°C with decreasing temperature. At 5°C it is about 100 sec^{-1} and rises again to about 450 sec^{-1} at 18°C. Above this temperature, the translocation rate increases to a lesser extent, while above about 25°C it decreases again. In principle the behavior of the translocation rate with $1/T$ can thus be interpreted in terms of two processes with different activation energies. The activation energy for the temperature range of 20 to 5°C is calculated from Eq. (4) to be 80 to 125 kJ/mol, while the activation energy for the upper temperature range is correspondingly found to be 4 to 9 kJ/mol. A similar behavior with T has been described by Thorhaug [22] for the membrane potential of *Valonia*.

The dependence of the electrical membrane conductivity L_m ($1/R_m$) upon $1/T$ is also found to be

Table 1. Temperature dependence of the different cell parameters of *Valonia utricularis* given for three different cells[a]

Cell No.	τ_1 (μsec)	τ_2 (msec)	T (°C)	k (sec^{-1})	R_m (Ωcm^2)	N_t ($pmol/cm^2$)
1	100	6.56	18	554	2571	7.21
	1062	14.38	5	71	6388	6.81
	128	5.82	18	481	2258	7.77
	668	5.96	34	232	4731	6.29
2	142	3.87	18	642	1996	5.30
	1586	8.85	5	72	19206	5.11
	103	3.58	19	697	1711	5.87
	531	5.71	34	485	3247	5.82
3	100	1.55	19	894	1096	3.10
	169	6.00	33	459	3216	5.18
	121	3.66	18	602	1114	3.02
	433	16.81	8	169	5773	2.52

[a] As can be seen, the changes in k, R_m and N_t are more pronounced in the low temperature range but remain fully reversible as long as the limits of the temperature range (5° and 34°C, respectively) are not transgressed.

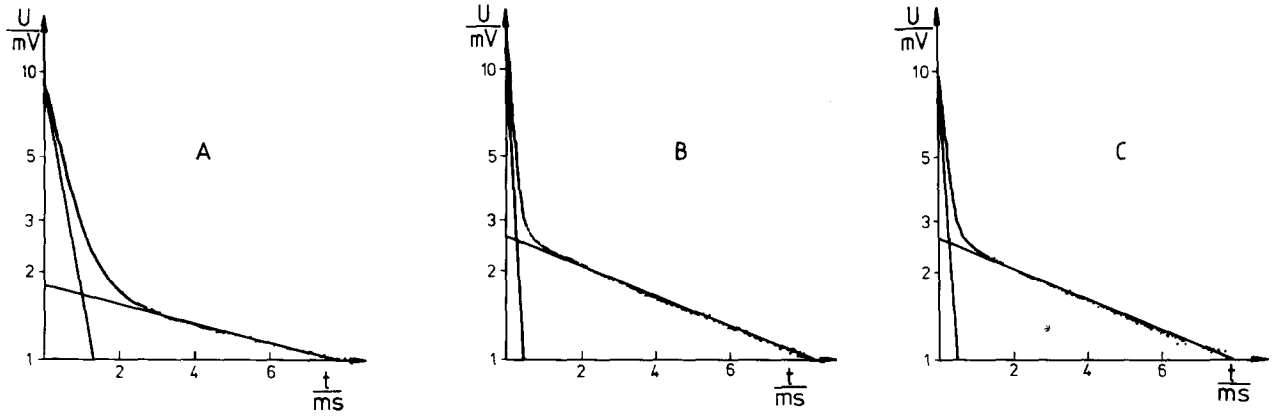

Fig. 1. Semilogarithmic plots of voltage relaxations of a typical cell of *Valonia utricularis vs.* time at 5°C (*A*), 18°C (*B*), and 34°C (*C*). Whereas there is a considerable increase in both time constants at 5°C (i.e. τ_1 = 500 μsec and τ_2 = 11 msec compared to τ_1 = 130 μsec and τ_2 = 5 msec in the control) the temperature dependence at higher temperatures (34°C) is less marked (τ_1 = 160 μsec and τ_2 = 6 msec)

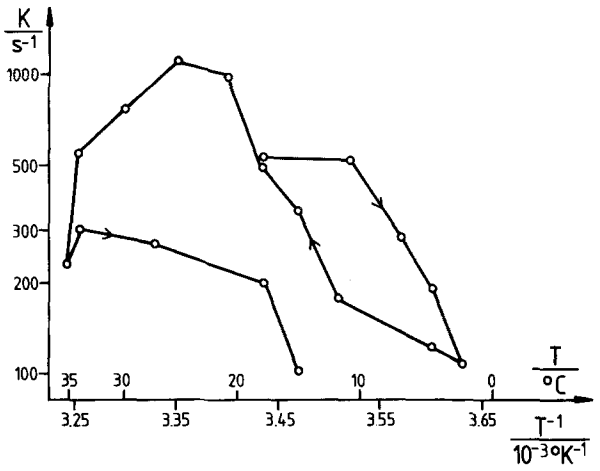

Fig. 2. Dependence of the translocation rate k of the mobile charges upon $1/T$. The direction of the temperature change is indicated on the graph by arrows. Note that there is a marked maximum of k around 25°C which could not be reproduced because of irreversible effects at high temperatures (35°C). The hysteresis effect occurring at low temperatures is also only reproducible as long as the permissive temperature range is not exceeded. For further explanations *see text*

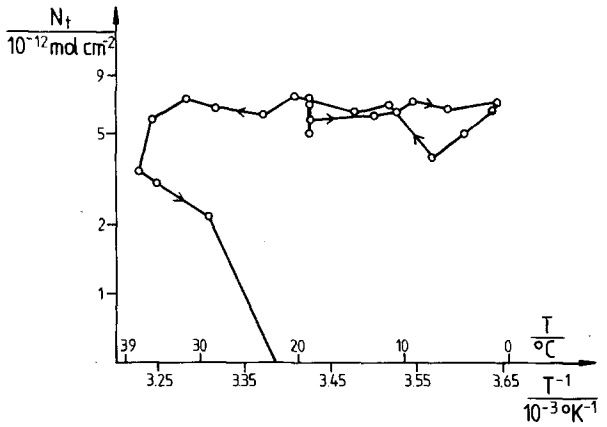

Fig. 3. Temperature dependence of the concentration N_t of the mobile charges of a cell of *Valonia utricularis*. No significant change in N_t could be detected over the whole temperature range between 5 and 34°C whereas an apparent sharp irreversible decrease occurred, when this temperature range was exceeded

similar to that of the translocation rate. The membrane conductivity reaches a peak value in the physiological temperature range. The concentration of the mobile charges is apparently independent of temperature in the range between 5 and 34°C (Fig. 3), which is consistent with the model assumption of an unsaturated carrier.

Figure 4 shows that hysteresis occurs following the cooling and subsequent warming of the seawater, i.e. during temperature increase to a value of about 20°C, the values for the translocation rate and membrane conductivity are found to be lower at the same temperatures than during the preceding temperature reduction. A corresponding hysteresis is observed when the temperature is first increased and subsequently reduced, although this is generally less marked than the hysteresis observed when the temperature is first reduced to lower values. However, this applies only if a temperature of about 34°C is not exceeded. Figure 2 shows that the translocation rate no longer assumes its original values at 18°C, if the temperature was initially raised to above 34°C. Under these conditions, irreversible changes take place in the membrane which no longer permit a recovery to the original translocation rate (and membrane conductivity, *not shown*). Similar irreversible changes in both parameters at room temperature were observed, if the temperature was initially reduced to below 5°C (*not shown*).

Above 34°C a marked reduction in the concentration of the mobile charges is observed. It decreases from a value of 5 pmol cm^{-2} (at 18°C) to a value of below 1 pmol cm^{-2}, assuming, that the model is still valid above 34°C and below 5°C (Fig. 3).

The hysteresis phenomena observed in the temperature range of 5 to 34°C are independent of the period of time during which the alga is maintained at the low or high temperature. In some algae it was possible to maintain temperatures of 5°C for more than 30 min without any change in the voltage relaxations and their time constants at 18°C.

In this respect the recovery behavior of the translocation rate and the membrane conductivity is analogous to that previously observed for pH changes [28]. When the pH was lowered from 8 to 5, one of the relaxations was seen to disappear within 10 min (probably caused by the neutralization of the negative mobile charges), while the reappearance of two relaxations, following an increase of the pH to pH 8, took about 1 hr. When the pH was lowered to pH 4 for longer periods of time, recovery of the two voltage relaxations and, in turn, of the original translocation rate was incomplete.

In a further set of experiments the influence of inhibitors of protein synthesis was investigated. No significant influence of chloramphenicol on the voltage relaxation patterns could be detected, compared to control experiments, neither at concentrations of 1 to 2 mg/ml nor at 10-fold higher concentrations. Addition of 0.1 or 0.2 mg/ml cycloheximide, however, led to a marked change in the relaxation constants. Table 2 shows the influence of cycloheximide as compared to the control recorded at various temperatures. As can be seen, the temperature dependence of the membrane conductivity

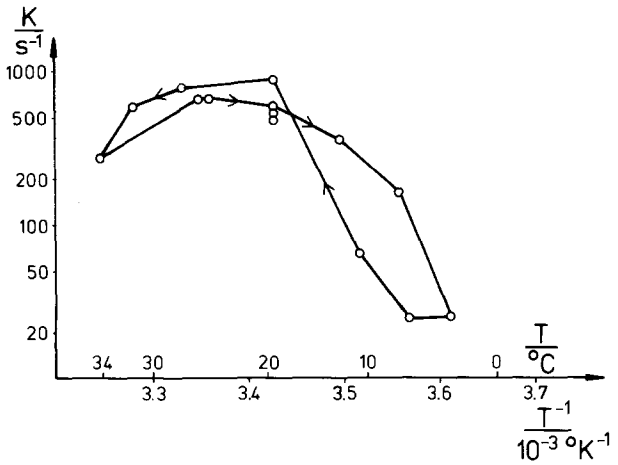

Fig. 4. Dependence of the translocation rate k of the mobile charges upon $1/T$ for an experiment in which the permissive temperature range was not exceeded. The hysteresis at low temperatures as well as hysteresis at high temepratures were both reproducible several times under these conditions. Temperature course is indicated by arrows, starting at 18°C with decreasing temperature

was more pronounced after cycloheximide treatment, leading to lower minimum values at 6 and 34°C. The effect of cycloheximide was only partially reversible, at least within several hours. In some experiments a slight decrease in turgor pressure by about 0.5 bar was observed after the addition of cycloheximide. This was compensated by a slight dilution of the external seawater.

Discussion

The experiments described in the present work were designed to test the possibility that the mobile charges within the membranes are coupled to the junction of an ion transport system, which consists of a carrier protein. We have therefore attempted to modify this hypothetical carrier function both by influencing the lipid micro-environment via temperature variations, and interfering with protein synthesis.

Membrane proteins involved in the transport or channeling of ions are integral proteins and, thus, would be in close interaction with the surrounding lipid layers. It seems therefore plausible that they will be affected by the physical state of the lipid micro-environment which, in turn, is a function of temperature and other factors. The temperature dependence of a series of electrophysiological properties, that reflect ionic movements through the cell membrane has been extensively studied in different plants like *Nitella* [12] and various animal systems.

Table 2. Influence of 0.2 mg/ml cycloheximide in ASW on the cell parameters of *Valonia utricularis* (cell No. 2) as compared to the control (cell No. 1)[a]

Cell No.	τ_1 (μsec)	τ_2 (msec)	T (°C)	k (sec^{-1})	L_m (Ω^{-1} cm^{-2} · 10^6)	N_t ($pmol/cm^2$)
1	1631	12.42	5	185	147	4.95
	82	4.64	20	788	384	5.61
	622	8.21	34	222	161	4.71
	103	5.07	20	645	351	5.49
2	3752	37.34	6	20	44	2.02
	109	10.72	20	713	162	5.73
	1967	22.02	33	33	87	0.59
	1290	20.91	20	143	167	0.28

[a] It should be noted, that after addition of the cycloheximide, the concentration of the mobile charges starts to decrease continuously.

In chick muscle [15], frog muscle [19], and locust muscle [2] as well as in neurons of *Aplysia* [21] and frog and rabbit nerves [13, 21] channel conductances have been measured as a function of temperature and the results are discussed as an indication for the occurrence of phase transitions in the boundary lipids, influencing the transport properties of the proteins involved, or, alternatively, as conformational changes in these proteins.

We have now demonstrated a qualitatively similar temperature dependence of the kinetic parameters characterizing mobile charge displacement in the membranes of *V. utricularis*. In particular, the translocation rate displays an about 10-fold decrease upon lowering the temperature from 18 to 5°C. Above 18°C the translocation rate increases only slightly, the net result being that these two temperature intervals, from 5 to 18°C and from 18 to 32°C, have very different activation energies, i.e. 100 ± 20 and 9 ± 4 kJ/mol, respectively. These results are consistent with the view that the mobile charges are part of, or coupled to, a carrier system, which is modulated by the degree of order of the lipid environment. No specific assignment of the break at 18°C to phase changes of a particular lipid domain can be made; however, the data show that mobile charge displacement which we suppose to precede or to accompany ionic transport, is subject to the same influences of the membranal micro-environment as those mentioned in the work cited earlier.

A further support for this interpretation is derived from our observation that the concentration of mobile charges remains practically constant over the entire temperature range from 5 to 34°C.

It is to be noted that the temperature variations of the membrane conductivity are analogous to

those of the translocation rate and, therefore, are in line with the work on other systems as well as with membrane potential measurements on *Valonia* carried out by Thorhaug [22].

The assumption that the postulated carrier involves a protein component is suggested also by the hysteresis effects within the temperature range monitored so far, as well as by the irreversible changes in translocation rate, conductivity and concentration of mobile charges when the limits of this range are transgressed. Provided the assumptions on which our model is based [28] are still valid, these results are consistent with partial protein denaturation, preceded by some metastable states, which influence one or several steps in the process of charge displacement. Such a denaturation would probably modify any conformational transitions and/or protein-lipid interactions, which are necessary for the charge movement.

Investigation of the temperature dependence of the electric breakdown voltage in cells of *V. utricularis* and *V. ventricosa*, as well a planar bilayers of oxidized cholesterol have shown an increase in breakdown voltage with decreasing temperature [5, 14]. This increase is particularly marked below 5°C, but nevertheless is still reversible. The breakdown is attributed to an electromechanical compression of the membrane [14] and so its increase at lower temperatures can be explained by an increase in the rigidity of the membrane (*see* Fig. 6 in ref. [10]). It thus seems that any irreversible structural changes occurring below 5°C, that have drastic consequences on the translocation rate of the mobile charges, membrane conductivity and the mobile charges' concentration do not affect the reversibility of electrical breakdown. According to available data [27] electrical breakdown occurs in the lipid domains and at the lipid protein interfaces, and, thus the differences in reversibility of the two phenomena can be readily understood.

The experiments with cycloheximide suggest that interfering with membrane protein synthesis considerably influences the translocation rate and membrane conductivity, which would also strengthen the concept of a carrier protein in the mobile charge phenomenon. However, the fact that the concentration of mobile charge remains unchanged may mean that these are not part of this protein, but molecularly different entities, utilizing the carrier. This carrier system could be operative in the transport of protons and/or potassium ions, which in *Valonia* has been shown to be turgor pressure dependent (*cf.* [28]). Alternatively, it could be assumed that a binding of the oligofunctional cycloheximide to the protein component influences the transport kinetics of the mobile charges.

It is of interest to mention in this context that Applewhite et al. [3] found that cycloheximide influenced rhythmic movement in *Albizzia julibrissin*, which is accompanied by rhythmic protein synthesis, and according to these authors is dependent on K^+ transport across the plant membrane. Hence the authors suggested that protein synthesis is involved in K^+ transport. Although Mummert and Gradmann [20] dispute a dominant role of K^+ pumping in turgor movements, we believe that the conclusions of Applewhite et al. [3] are likely, since direct measurements of turgor pressure in algal cells have shown a number of membrane processes to be regulated by pressure [16, 25, 26]. Läuchli et al. [18] have also demonstrated inhibition of K^+ transport through barley roots by cycloheximide.

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