# CHLORIDE INFLUX INTO CITRUS LEAF SLICES

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### Summary

The influx of  ${}^{36}$ Cl has been measured in slices of citrus leaves. The influx increases with increasing external chloride concentration. The shape of the influx v. concentration curve depends on the nature and concentration of the accompanying cations (Na<sup>+</sup> and K<sup>+</sup>). There is no evidence for the two distinct chloride transport systems reported by other workers. Chloride influx was not stimulated by light under aerobic conditions and was sensitive to 2,4-dinitrophenol at uncoupling concentrations. Under anaerobic conditions influx was maintained by light but severely limited by dark conditions. 3-(chlorophenyl)-1,1-dimethylurea did not affect aerobic influx and only high levels ( $5 \times 10^{-5}$ M) affected the anaerobic influx in the light. It is proposed that chloride influx is normally dependent on oxidative phosphorylation but that cyclic photophosphorylation may provide an alternative energy source. The implications of these results are discussed with respect to ionic relations of whole leaves and salinity damage.

## I. INTRODUCTION

Leaf material is often sampled and analysed as a means of assessing the state of the whole plant with respect to nutrient and of course detrimental ions (e.g. Chapman 1966). Such techniques must consider the ionic relations of the leaf cells as a balanced equilibrium system. However, when transpiration is taking place, ions must constantly enter the free space (Briggs and Robertson 1957) of the leaf. Some understanding is needed of the dynamics of the ion levels in the free space with relation to transpirational inputs and movement into and out of the cellular compartment, if we are to understand fully the dynamics of ions in the whole plant.

Most studies of ion transport in higher plant tissue have involved the use of excised tissue, either aged for some time or taken from plants grown under welldefined but physiologically abnormal conditions. Attempts to extrapolate to the native state data obtained with such material may be misleading.

In the present study with citrus leaf tissue collected from the field, the time between sampling and experiment has been kept to a minimum. The aim of the work is to give quantitative values to some of the parameters necessary to describe the leaf as a dynamic system. This paper describes measurements of chloride uptake in relation to the external concentration of chloride and accompanying cations. The influence of sodium ions on chloride uptake has been examined in some detail and is discussed in relation to current theories of salinity damage to leaf tissue. Links between metabolism and chloride uptake have also been briefly studied.

Fluxes of cations are at present under investigation and will be described in a subsequent paper.

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## II. MATERIALS AND METHODS

Leaves used were from a sweet orange [Citrus sinensis (L.) ev. Valencia] tree growing in an Adelaide garden. They were brought to the laboratory over ice. Slices 800–900  $\mu$ m wide were cut and placed into a large volume of deionized water and rinsed twice to remove ions released from damaged cells. Slices of this thickness had essentially the same uptake characteristics as thinner slices and were considerably more convenient to prepare and handle. Slices were randomly allocated to batches (approximately 0.5 g), blotted, and placed into 10 or 25 ml of <sup>36</sup>Cl-labelled solution for 2 hr. The slices were then removed, blotted, and placed in deionized water for 5 min to wash out radioactivity from the free space. The tissue was then thoroughly blotted, weighed, and placed in boiling-tubes with 10 ml deionized water. These tubes were covered and placed in a boiling water-bath for longer than 1 hr. After cooling, duplicate aliquots were transferred to planchets, dried, and the radioactivity was assayed. Chloride influx was calculated as  $\mu$ moles per gram fresh weight per hour.

Experiments were carried out at room temperature  $(22-28^{\circ}C)$  except where specified. Light was provided by four 20 W fluorescent tubes at a distance of 30 cm from the tissue. The tubes were aerated throughout the uptake period. In some experiments nitrogen was substituted for air.

The standard experimental solutions contained  $0.5 \text{ mM} \text{ CaSO}_4$  and sodium chloride at concentrations between 0.1 and 100 mM. Actual concentrations of sodium chloride, potassium chloride, sodium sulphate, and mannitol in individual experiments are specified below. All reagents were analytical grade. The metabolic inhibitors 2,4-dinitrophenol (DNP) and 3-(chlorophenyl)-1,1-dimethylurea (CMU) were used in some experiments. <sup>36</sup>Cl was obtained from the Radiochemical Centre, Amersham, U.K.

Respiration was measured using conventional Warburg manometers at 25°C. Tissue samples weighing 0.2-0.3 g were used in these experiments.

### III. RESULTS

## (a) General

Preliminary experiments showed that uptake of  ${}^{36}$ Cl from 10 mM NaCl and  $0.5 \text{ mM} \text{ CaSO}_4$  was linear over the 2 hr uptake period, and that the 5 min rinse in deionized water removed well over 95% of the  ${}^{36}$ Cl held in the free space of the tissue. The experiments described below were carried out between mid-November 1969 and late January 1970. It was found that influx of chloride under standard conditions varied little over this period. Mature leaves from the spring 1969 growth flush were normally used and gave mean influxes in light and dark of  $1 \cdot 15 \pm 0 \cdot 06(17)$  and  $1 \cdot 16 \pm 0 \cdot 05(15) \mu$ moles per gram fresh weight per hour respectively (values quoted are the mean  $\pm$  standard error of the mean and the number of experiments is given in parenthesis) from solutions containing 10 mM NaCl and  $0.5 \text{ mM} \text{ CaSO}_4$ . In one experiment chloride uptake into slices of expanding leaves from the summer 1969–70 growth flush was compared with the uptake into the mature leaves, and leaves from an earlier flush. The chloride influxes obtained for orange leaf slices of varying ages are shown in the following tabulation:

	Chloride Influx [ $\mu$ moles (g fresh wt.) <sup>-1</sup> hr <sup>-1</sup> ]		
	Light	Dark	١
Young expanding leaves, summer 1969–70 flush	$1 \cdot 32$	$1 \cdot 20$	
Mature leaves, spring 1969 flush	$1 \cdot 08$	$1 \cdot 31$	
Old leaves, from flush prior to spring 1969	0.65	0.67	

The leaves were sampled sequentially along a single shoot. The results show that chloride uptake remains relatively constant during much of the life of the leaf.

## (b) Concentration Effects

Chloride influx was measured over a concentration range 0.1-100 mM NaCl and was found to increase with increasing concentration. Typical results from such an experiment are presented in Figure 1. Over the low concentration range (0.1-1 mM)



Fig. 1.—Effects of external sodium chloride concentration on the chloride influx in light. All solutions contained  $0.5 \text{ mm CaSO}_4$ . The low (a) and high (b) concentrations are plotted separately. Fig. 2.—Effects of external sodium concentration on the chloride influx in light. Basic solution contained  $10 \text{ mm NaCl}+0.5 \text{ mm CaSO}_4$ . Sodium was added as sodium sulphate.

the influx increased linearly with increasing concentration, and even with high concentrations (30 and 100 mm NaCl) there was little saturation of the influx (see also Fig. 3). The shape of the influx isotherm below 0.1 mm NaCl was not investigated.



Fig. 3.—Effects of sodium level on chloride influx over increasing concentrations. Two experiments, (a) and (b), are plotted. Sodium was held constant at 100 mM in light ( $\Box$ ) and dark ( $\blacksquare$ ), or added as sodium chloride in light ( $\bigcirc$ ) and dark ( $\bullet$ ). All solutions contained 0.5 mM CaSO<sub>4</sub>. Sulphate was used as counter-ion where sodium was maintained at a fixed level.

It was found that chloride influx at a given concentration was greatly influenced by the external sodium concentration. As the sodium concentration was increased from 10 to 610 mm by the addition of sodium sulphate (chloride held constant at 10 mm) the influx of chloride also increased (see Fig. 2). When the sodium concentration was held at 100 mm and the chloride concentration varied from 1 to 100 mm the shape of the influx isotherm was altered. This difference is shown in Figure 3.

At the higher concentrations of sodium shown in Figure 2, the osmotic pressure of the solution would be very high. However, raising the osmotic pressure with mannitol did not affect chloride influx from 10 mM NaCl. The combined data from two experiments are plotted in Figure 4. It should also be noted that no alterations in respiration rate were brought about by sodium sulphate up to 300 mM or mannitol up to 600 mM.



That the external cation level limits chloride influx is suggested by the above results, and by experiments in which potassium sulphate was added to the bathing media to give potassium concentrations ranging from 0 to 100 mm, with 10 mm NaCl and 0.5 mm CaSO<sub>4</sub> also present. Again the chloride influx increased as the cation concentration rose (Fig. 5).



Fig. 5.—Effect of increasing potassium level on chloride influx in light  $(\odot)$  and dark  $(\bullet)$ . The basic solution contained 10 mm NaCl+0.5 mm CaSO<sub>4</sub>. Potassium was added as potassium sulphate.

Fig. 6.—Effect of increasing chloride concentration on chloride influx, where potassium concentration was maintained at 100 mM, in light ( $\odot$ ) and dark ( $\bullet$ ). The basic solution contained 10 mM sodium + 0.5 mM CaSO<sub>4</sub>. Sulphate was used as counter-ion.

The experiment shown in Figure 6, where potassium was maintained at 100 mm and the chloride concentration was increased from 1 to 100 mm, should be compared with the upper curves shown in Figure 3. The potassium levels needed to increase chloride influx were lower than the sodium levels needed to give a similar influx (compare also Figs. 2 and 5).

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## (c) Metabolic Effects

Chloride influx was reduced by low temperature over a wide range of external chloride concentrations (see following tabulation) suggesting that the influx is under metabolic control:

Chloride Concn. (mM)	Chloride Influx [ $\mu$ mole (g fresh wt.) <sup>-1</sup> hr <sup>-1</sup> ]		Chloride Concn. (mM)	Chloride Influx [ $\mu$ mole (g fresh wt.) <sup>-1</sup> hr <sup>-1</sup> ]	
	22°C	7°C	(11111)	΄ 22°C	7°C
$0 \cdot 1$	0.023	0.004	10	0.97	0.160
0.3	0.066	0.013	30	$3 \cdot 42$	0.272
1	$0 \cdot 147$	0.024	50	$4 \cdot 67$	0.383
3	$0 \cdot 40$	0.059			

The influx was also inhibited by concentrations of DNP which uncoupled respiration (Fig. 7). In most experiments where both light or dark conditions were used there was no difference [e.g. Figs. 3(b), 5], although small increases were sometimes found (e.g. Figs. 6, 8). As noted in Section III(a), when all the experiments carried out under



Fig. 7.—Effect of DNP on chloride influx  $(\bigcirc)$  and oxygen consumption  $(\Box)$  in the dark. The basic solution in both cases contained 10 mm NaCl+0.5 mm CaSO<sub>4</sub>.

Fig. 8.—Effect of CMU on chloride influx under aerobic conditions, in light ( $\bigcirc$ ) and dark ( $\bigcirc$ ) and under nitrogen in light ( $\square$ ) and dark ( $\blacksquare$ ). The basic solution contained 10 mm NaCl+0.5 mm CaSO<sub>4</sub>.

standard conditions were pooled there was no difference. This does not provide any evidence for a linkage between chloride influx and photosynthetic metabolism. The effects of DNP suggest a linkage to oxidative phosphorylation. However, this linkage does not appear to be obligatory; although under anaerobic conditions influx was reduced by 90% in the dark, no reduction occurred in the light (see following tabulation in which chloride influxes are expressed as  $\mu$ moles per gram fresh weight per hour):

	$\mathbf{Light}$		Dark	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Air bubbled	$1 \cdot 10$	$1 \cdot 59$	1.00	$1 \cdot 62$
Nitrogen bubbled	0.95	$1 \cdot 74$	$0 \cdot 12$	0.18

These results suggested that cyclic photophosphorylation can provide an alternative energy supply. This is supported by the finding that a high concentration of CMU (which prevents non-cyclic electron flow) was needed to reduce chloride influx in the light under nitrogen (Fig. 8).

# IV. DISCUSSION

## (a) Concentration Effects and Chloride Influx

As a result of studies with plant material grown under low salt conditions, Epstein and co-workers (Epstein 1966) and Laties (1969) have proposed salt transport mechanisms in higher plants involving two systems, a high affinity system (I) operating at low external salt concentrations and a low affinity system (II) operating at concentrations in excess of 1 mm. These are based on interpretation of the shape of the absorption isotherm, system I saturating at low concentrations, up to 1 mm, and system II saturating at 10-30 mm. It has been suggested that this pattern "may be virtually universal in mature tissues of higher plants" (Epstein 1969). Our work provides no evidence for this type of concentration dependence in the case of chloride in citrus leaves. We have shown that the shape of the chloride isotherm depends on the level of cations (Na<sup>+</sup> or K<sup>+</sup>) present in the bathing medium and believe that any attempt to interpret these isotherms in terms of the uptake of chloride per se would be unrealistic. There are several ways in which cations and chloride might interact. Changes in cation levels would be expected to alter permeability and electrical properties of the cell membrane. These in turn might affect chloride transport. There might also be direct effects on the chloride transport system if this is dependent on the presence of cations, i.e. if chloride is transported by a neutral salt pump. The possible significance of such interactions has been ignored in most of the published work on ion transport in higher plant tissues.

### (b) Chloride Uptake and Metabolism

In order to gain some understanding of the links between chloride uptake and metabolism we chose a standard experimental solution containing  $10\,\mathrm{mm}\,\mathrm{NaCl}{+}0.5\,\mathrm{mm}$ CaSO<sub>4</sub>. Chloride uptake was extremely sensitive to lowered temperature, but was not increased in the light. Light-dependent chloride transport is common in algae and has been described in the leaves of the higher plants Vallisneria (Van Lookeren Campagne 1957) and Elodea (Jeschke 1967; Weigl 1967), but is absent in Atriplex (Osmond et al. 1969). The inhibition of influx by DNP at uncoupling concentrations suggests that oxidative phosphorylation normally powers chloride transport, although the inhibition could be due to a direct effect on the membrane system. However, under nitrogen the light influx rate is maintained, which may be interpreted as the use of cyclic photophosphorylation as an alternative energy supply. A high level of CMU  $(5 \times 10^{-5} \text{M})$  under nitrogen is needed to inhibit the light-dependent influx. This may reflect an inhibition of cyclic as well as non-cyclic electron flow. (A detailed study of the biochemical effects of CMU on citrus leaf cells would be needed to establish this point.) Jeschke (1967) and Weigl (1967) have suggested that chloride uptake in *Elodea* can be supported by cyclic phosphorylation and this appears to be so under anaerobic conditions in Citrus. When considering green tissue, in general, no clear picture can be gained. For example, in some freshwater algae non-cyclic electron flow appears to be necessary to support chloride influx in light (MacRobbie 1965; Raven 1967), but in others photophosphorylation has been implicated (Barber 1968; Smith and West 1969).

# (c) General

Under natural conditions the ionic environment of the individual leaf cells is provided by the free space within the leaf. We are hampered in attempting to describe the ionic relations of leaf cells by absence of reliable information about the size and ionic content of the free space of the leaf. Attempts have been made to estimate its ionic content by washing-out experiments with freshly cut leaf slices, but contamination from cut cells could not be ruled out (Robinson, unpublished data). Under these circumstances it is worth while to work with freshly prepared tissue slices and solutions with a concentration range wide enough to encompass expected levels in the free space. We feel that chloride levels from 10 mM upwards are most relevant to the natural situation. Where plants are subjected to high salinity there will be a considerable input of sodium chloride to the leaf via the transpiration stream which will increase the sodium chloride concentration in the free space and (depending on the rate of membrane transport) in the cells. Both free space and the cellular compartment will have an increased osmotic pressure. Bernstein (1961) has measured an increase in osmotic pressure of sap expressed from plants grown on saline media, and believes that such osmotic adjustment (attributed to the cellular compartment) will enable maintenance of turgor and growth of plants under salt stress. Our results suggest that there is increasing movement from the free space to the cellular compartment of the leaf with increasing external sodium chloride concentrations, which would contribute to the osmotic adjustment. However, as Oertli (1968) has pointed out, under conditions of high salinity, the input of sodium chloride into the attached leaf may exceed the movement from free space to the cellular compartment, and under extreme conditions the osmotic pressure of the free space may become high enough to account for salinity damage.

Both sodium and chloride have been assigned the role of the toxic ion by proponents of the "specific ion toxicity" theory of salinity damage (see Bernstein and Hayward 1958). In citrus leaf slices sodium level has a marked effect on the influx of chloride from solutions containing up to  $30 \text{ mm Cl}^-$ . On this evidence both sodium and chloride must be considered as interrelated in their entry to the cellular compartment. There are a number of ways in which these ions might affect membrane properties and metabolism (see Slatyer 1967). To discuss these in detail would be beyond the scope of this paper.

There is one further point of horticultural interest—where irrigation water containing dissolved sodium chloride has been sprayed on to the leaves of citrus trees it has been noted that the onset of salinity damage is slower when irrigation is carried out at night (Eaton and Harding 1959). Our data suggest that the increased sodium chloride content of the leaves in daytime is not caused by a direct effect of light, stimulating uptake into the cells. It is more likely that other micro-environmental factors (humidity, leaf temperature, concentration of ions at the leaf surface, etc.) are involved.

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