IONIC RELATIONS OF CELLS OF *CHARA AUSTRALIS*

XI.* CHLORIDE FLUXES

By H. G. L. COSTER† and A. B. HOPE‡

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Summary

Influx and efflux of chloride ions in *Chara australis* have been measured under a variety of conditions in an attempt to relate the active transport inwards to a particular aspect of metabolism. Influx across the plasmalemma in single cells was 1–4 p-equiv. cm^{-2} sec^{-1}. The rate constant for exchange of cytoplasmic chloride was of the order of 10^{-3} sec^{-1} and the content of chloride in the cytoplasm was found to be about 100 n-equiv. cm^{-2}. From these values the influx across the tonoplast was inferred to be $\simeq 100$ p-equiv. cm^{-2} sec^{-1}. Most of the cytoplasmic chloride was probably in the chloroplasts.

The effects of several reputed inhibitors of electron transport and photophosphorylation were studied in relation to active chloride influx at the plasmalemma. The action of these inhibitors is discussed in relation to MacRobbie's hypothesis that active anion transport is intimately connected with electron transport in chloroplasts while ATP production is more closely concerned with cation transport. The action of phlorizin in the present study was found to be inconsistent with the hypothesis if it is accepted that phlorizin is a selective inhibitor of photosynthetic phosphorylation.

The efflux of chloride and its contribution to the electrical conductance of the plasmalemma were found to increase greatly as this membrane was hyperpolarized. This is consistent with a model for the membrane which contains an abrupt junction between positive and negative fixed-charge regions.

I. INTRODUCTION

Earlier considerations (MacRobbie and Dainty 1958; Hope and Walker 1960) have strongly suggested that the influx of chloride in the giant internodes of characean cells is active in that the metabolism of the cell must provide energy for ions to be taken into the vacuole, against both an activity gradient and an electrical potential gradient. Conversely the efflux of chloride is passive and "downhill".

MacRobbie (1962, 1964, 1965, 1966*a*, 1966*b*) has made extensive studies of the connection between metabolic factors and active transport of Cl^- and K^+. The distribution of chloride between the chloroplasts, flowing cytoplasm, and vacuole has been examined by Kishimoto and Tazawa (1965) and Coster (1966), while Hope, Simpson, and Walker (1966) have given an account of the efflux of chloride under various conditions in terms of membrane permeability.

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† School of Physics, University of New South Wales, Kensington, N.S.W. 2033.

‡ School of Biological Sciences, Flinders University, Bedford Park, S. A. 5042.

MacRobbie (1965, 1966*a*, 1966*b*) has concluded that the active chloride influx at the plasmalemma in *Nitella translucens* is linked to electron flow during photosynthesis and is independent of ATP. The effects of the metabolic inhibitors carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), imidazole, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), *N*,*N*′-dimethyl-4,4′-dipyridylium dichloride (Paraquat), azide, and phlorizin on chloride transport are reported here and discussed in relation to MacRobbie's hypothesis. The passive efflux of chloride has been measured over a wide range of values of the vacuolar potential difference and estimates of the partial electric conductance due to chloride ions have been made over this range.

II. Methods

(*a*) *Influxes*

Estimation of influx was based on counting the radioactivity in whole internodes of *Chara australis* or in vacuolar sap and cytoplasm separately. Surface radioactivity and more than 99% of the cell-wall chloride was exchanged (Dainty and Hope 1959) by rinses of 1–5 min in inactive standard artificial pond water containing 0·1 mN KCl, 1·0 mN NaCl, 0·2 mN $CaCl_2$.

When the whole cell was to be counted the cell was dispersed into about 0·5 ml distilled water on a planchet, the nodes discarded, and the remainder dried and counted in a low-background, gas-flow counter. In some earlier experiments counting was done in a liquid-scintillation counter after drying the samples on segments of filter paper in standard scintillation vials and adding 2 or 5 ml scintillation liquid. Efficiency in both methods for ^{36}Cl was high (48–70%) but the background was <2 counts/min in the gas-flow counter compared with 20–25 counts/min in the liquid-scintillation method.

(*b*) *Relative Radioactivity of Vacuole and Cytoplasm*

(i) *Perfusion Method.*—Following the general method of Tazawa (1964), internodes were slowly perfused with approximately isotonic mannitol (0·25M) or other solutions to remove vacuolar sap without disturbing the cytoplasm. Both "fast" and "slow" perfusions were done, taking approximately 20 and 120 sec, respectively, to pass about five cell volumes of perfusion medium through the vacuole. If the perfusion time was reduced from 120 to 20 sec the chloroplast layer remained intact but some cytoplasm was removed (cf. Kishimoto and Tazawa 1965).

(ii) *Sap Sampling Method.*—Almost pure vacuolar sap, usually 10 μl in volume, could be obtained within a few seconds of cutting the cell. With knowledge of the total cell volume, the radioactivity in the sap sample and in the remainder, the proportion of activity in the cytoplasm could be estimated.

(*c*) *Chloride Analyses*

Electrometric titration using $AgNO_3$ was used to determine the chloride content in sap or cytoplasm samples. The method could detect as little as 50 n-equiv. with about 95% accuracy.

(*d*) *Effluxes*

The general method of determining efflux of Cl^- was that described by Hope, Simpson, and Walker (1966). After the labelling and rinsing period, cells had microelectrodes inserted in them for measurement of electrical potential (p.d.) and for passing current to modify the p.d. The efflux into stagnant artificial pond water was measured in periods where the p.d. was set ("clamped") at various levels. The time periods varied from 30 min near the resting potential to about 1 min when the p.d. was made very negative by a large current. Electric currents across

the cell membrane tend to modify the ionic concentrations near the membrane (Barry and Hope, unpublished data). At very high negative potentials the efflux was high enough to allow short collection periods during which the effects of current were relatively small. Details of the electrophysiological techniques have appeared previously (e.g. Walker 1960).

III. Results

(*a*) *Effect of Excision from Growing Plant*

Influxes of chloride ions over periods of 1–4 hr were measured in cells 2–5 cm long cut from growing plants, from the same relative position in each plant to reduce variability. The cells were trimmed and left in artificial pond water for various times.

Influx was calculated from

$$\phi = \frac{\Delta Y}{\Delta t} \cdot \frac{1}{S_o} \cdot \frac{1}{A},$$

where ΔY is the radioactivity appearing in the whole internode (nodal cells discarded) in time Δt, S_o is the specific activity of the artificial pond water, and A the surface area of the cell. This is a good estimate of ϕ_{oc}, the influx across the plasmalemma, as discussed below.

Table 1 lists the mean influx of chloride ions in groups of such cells for various times after excision. It is apparent that influx drops sharply after cells are removed from the whole plant but that an average influx of about 1 p-equiv. cm^{-2} sec^{-1} is maintained over periods of up to 14 days isolation. It was found that excised cells do not elongate appreciably following excision.

Table 1

Effect of time after excision (days) on chloride influx

Number of determinations are given in parenthesis

Date of Expt.	Chloride Influx (p-equiv. cm^{-2} sec^{-1}) 0 Day	1 Day	2 Days	3 Days	7 Days	14 Days
October 1965	2·0±0·4(4)	0·59±0·16(7)	0·93±0·18(7)	1·19±0·23(7)		
	1·73±0·17(9)					
June 1966	3·2±0·4(11)					
September 1966	2·8±0·4(8)	1·91±0·09(9)				
		1·75±0·2(10)				
June 1967	4·6±0·1(9)				1·6±0·5(10)	1·6±0·2(11)

(*b*) *Chloride Content of the Cytoplasmic Phase*

A measure of the amount of chloride in the cytoplasm (flowing cytoplasm + gel layer + chloroplasts) was needed for the estimation of relative fluxes at the tonoplast and plasmalemma.

The chloride content of cells pretreated with artificial pond water, after perfusion with mannitol and other solutions tinged with neutral red, are presented

below. The number of determinations are give in parenthesis.

Perfusion Medium	Perfusion Speed	Cl^- Content (n-equiv. cm^{-2})
Mannitol, 0·2M	Slow	74±10(5)
Mannitol, 0·2M	Fast	88±15(4)
Mannitol, 0·25M*	Slow	87±15(4)
Mannitol, 0·20M + $Ca(NO_3)_2$, 0·01M	Slow	96± 9(6)
$Ca(NO_3)_2$, 0·075M	Slow	86±18 (5)

* Pretreated with artificial pond water +0·5 mM $NaNO_3$.

Considering the likelihood of a thin layer of vacuolar sap, with $[Cl^-] \simeq 100$ mN remaining in the perfused "cells", it is thought that the lower values are the more reliable, i.e. 80–90 n-equiv. cm^{-2}. The value of 90 n-equiv. cm^{-2} is used below in estimating fluxes from rate constants for cytoplasmic chloride turnover.

To convert ionic quantity to concentration, the volume of the cytoplasmic phase must be estimated. Cells comparable with those used in the estimation of chloride per unit area were centrifuged in tubes with capillary bases at $\simeq 3000\ g$ so that the cytoplasm and chloroplast became layered below the vacuolar sap. From volumes of chloroplast pellet layer and clear cytoplasm layer obtained in this way actual volumes can be derived: if it is assumed that at $\simeq 3000\ g$ the chloroplasts are packed with about 50% interparticulate volume, then actual chloroplast volume would be half the pellet volume and the cytoplasm volume would be correspondingly greater. Volumes (μl cm^{-2}) of the chloroplast layer ($A_{est.}$) and cytoplasm ($B_{est.}$) estimated from volumes of chloroplast pellet (A) and clear cytoplasm (B) for a number of cells are shown below.

A	B	A+B	$A_{est.}$	$B_{est.}$
0·40	0·20	0·60	0·20	0·40
0·35	0·15	0·50	0·17	0·33
0·45	0·15	0·60	0·22	0·38
0·65	0·55	1·20	0·32	0·88
0·45	0·15	0·60	0·22	0·38
—	0·60	—	—	0·60
—	0·70	—	—	0·70
—	—	0·65	—	—
—	—	0·40	—	—

Mean volumes (μl cm^{-2}) ±S.E. for A+B (total cytoplasmic volume), $B_{est.}$, and $A_{est.}$ are 0·65±0·14, 0·52±0·1, and 0·22±0·02 respectively. This value for A+B corresponds to an average thickness of 7 μ which is reasonable. The value of 10 μ often quoted is usually based on an impression of the depth of focus in the cytoplasm when observed under a microscope.

(c) *Relative Fluxes at the Plasmalemma and Tonoplast*

The methods of perfusion and of vacuolar sap sampling were used to gain a measure of the proportion of radioactivity found in the cytoplasm compared with the cell total following short labelling periods. This proportion is related to the rate constant k_c for turnover of cytoplasmic chloride, which in turn is equal to

$(\phi_{co}+\phi_{cv})/Q_c$, where Q_c is the amount of chloride in the cytoplasm per square centimetre of cell surface.

A comparison of the mean plasmalemma influxes (ϕ_{oc}, p-equiv. $cm^{-2}\,sec^{-1}$), mean cytoplasm rate constants (10^3k_c, sec^{-1}), and the sum of fluxes out of the cytoplasm ($\phi_{co}+\phi_{cv}$) under different conditions is made below. Cells were from Ross, Tasmania; number of determinations are given in parenthesis.

Date of Expt.	Conditions	ϕ_{oc}	10^3k_c	$\phi_{co}+\phi_{cv}$
11.iv.66	Light, 21·5°C	2·7±0·6(6)	1·7±0·4(6)	160
18.iv.66	Dark, 25°C	0·10±0·03(6)	0·64±0·14(6)	60
21.iv.66	Light, 25°C	1·5±0·35(7)	1·6±0·3(7)	140
29.iv.66	Dark or dim light, 22°C	0·23±0·07(6)	1·0±0·3(7)	90

Figure 1 shows k_c plotted against ϕ_{oc} for the various experiments. The two quantities seem to be correlated. Details of the method of calculating the fluxes from the observations are given below.

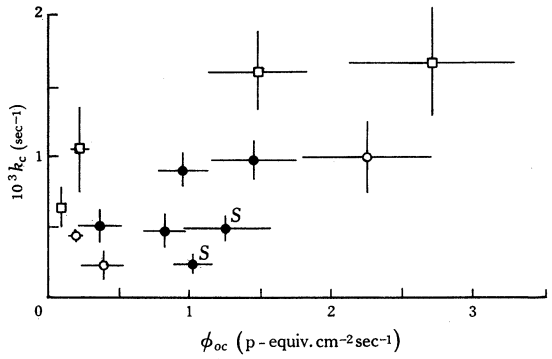

Fig. 1.—Influx of chloride across the plasmalemma (ϕ_{oc}) plotted against the rate constant for chloride exchange in the cytoplasm (k_c). The different symbols refer to cells of different origin or culture method. k_c values were obtained by the perfusion method except in those cases denoted by *S* where k_c was estimated from radioactivity in sap samples.

(*d*) *Effects of Inhibitors*

After pretreatment for 30–60 min in artificial pond water with the appropriate inhibitor added, cells in batches of 8–10 were placed in the corresponding radioactive solutions. The mean influxes of Cl^- in several such solutions are listed in Table 2. Cells were inspected for damage after the influx period. For example, absence of cytoplasmic streaming was a sure prediction of early death; non-streaming cells are indicated in the table.

Reversibility of inhibitor effects on chloride influx was tested by pretreatment with inhibitor, followed by 1 hr in artificial pond water and a subsequent labelling period using radioactive artificial pond water without any inhibitors. Results of these experiments are given below. Number of determinations are given in parenthesis.

Conditions of Expt.	Chloride Influx (p-equiv. $cm^{-2}\,sec^{-1}$)	Influx as % of Control	*P*
Artificial pond water	0·83±0·13(10)	100	
DCMU, 3 μM	0·38±0·09(10)	46	<0·01
Phlorizin, 0·1 mM	0·57±0·085(10)	69	<0·05
Paraquat, 0·1 mM	0·37±0·07(10)	45	<0·01

(e) *Efflux and Electrical Conductance*

Chloride effluxes were measured at various potentials by the method described. The accumulated data for seven cells are shown in Figure 2. It can be seen that the efflux is small near the resting potential, which was about −150 mV, but increases

TABLE 2

EFFECT OF METABOLIC INHIBITORS ON CHLORIDE INFLUX

Numbers of determinations are given in parenthesis

Inhibitor	Concn. of Inhibitor	Date of Expt.	Chloride Influx (p-equiv. cm^{-2} sec^{-1}) Control	Control + Inhibitor	Influx as % of Control
Azide	0·5 mM	8. x.65	2·0 ±0·4(4)	0·82±0·12(6)	41**
		12. x.65	1·19±0·24(7)	0·57±0·21(7)	48**
		14. x.65	0·93±0·18(7)	0·32±0·21(8)	34**
		15. x.65	1·73±0·17(9)	1·64±0·23(10)	95
DCMU	3 μM	14.vi.66	3·20±0·4(11)	0·70±0·29(4)	22**
	0·3 μM	26.ix.66	1·91±0·09(9)	1·72+0·38(9)	90
	1 μM			0·98±0·14(9)	51**
	3 μM			1·05±0·20(9)	55**
	3 μM	11. ii.67	0·85±0·14(10)	0·34±0·04(9)	40**
Paraquat	0·05 mM	15.vi.66	3·20±0·4(11)	0·72±0·21(5)	22**
	0·10 mM			0·58±0·05(4)	18**
	0·04 mM	26.ix.66	1·91±0·09(9)	1·57±0·17(9)	82
	0·10 mM	30.ix.66	1·75±0·20(10)	1·59±0·29(9)	91
	0·10 mM	11. ii.67	0·85±0·14(10)	0·27±0·05(8)	32**
CCCP	1 μM	26.ix.66	1·91±0·09(9)	1·76±0·14(9)	92
	3 μM			0·31±0·05(9)	16**
	10 μM†			0·08±0·01(12)	4**
	2 μM	30.ix.66	1·75±0·20(10)	1·97±0·20(9)	112
	2 μM‡	11. ii.67	0·85±0·14(10)	0·30±0·11(9)	35**
	2 μM§			0·70±0·07(11)	82
Phlorizin	0·03 mM	26.ix.66	1·91±0·09(9)	0·97±0·11(9)	51**
	0·1 mM			0·94±0·15(9)	49**
	0·1 mM	11. ii.66	0·85±0·14(10)	0·24±0·02(8)	28**
Imidazole	0·1 mM	15. ii.67	1·15±0·14(13)	1·58±0·15(10)	137*
	1·0 mM†			0·22±0·04(10)	19**

* Mean values significantly different from control at $P < 0·05$.

** Mean values significantly different from control at $P < 0·01$.

† Cells not streaming at end of influx period.

‡ Light conditions during influx period.

§ Dark conditions during influx period.

very steeply in the direction of hyperpolarization. The average resting influx for these cells was 0·33 p-equiv. cm^{-2} sec^{-1} and, assuming that the influx is constant, the chloride conductance can be calculated as a function of membrane potential

since $g_{Cl} = F(\partial\phi_{net}/\partial E)$. This is shown in Figure 3. Some function such as the line drawn through the points in Figure 2 has to be assumed to connect efflux and potential to enable this to be done. It can be seen that the chloride conductance, g_{Cl}, is very low (2·6 μmho cm^{-2}) at the resting potential but rises very steeply to 940 μmho cm^{-2} at −350 mV.

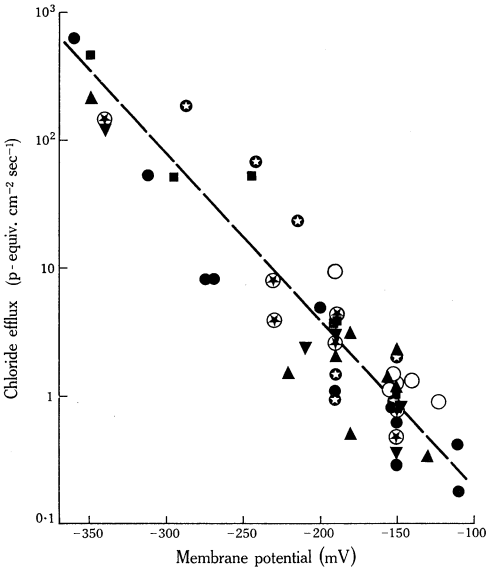

Fig. 2

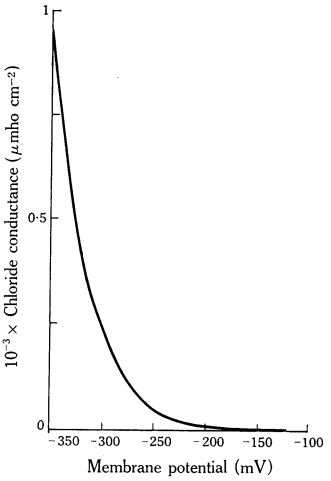

Fig. 3

Fig. 2.—Accumulated data of Cl^- effluxes plotted against membrane potential for seven cells. The dashed line was used as a basis for the calculation of g_{Cl} *v.* potential (see Fig. 3). The average resting influx of Cl^- for these cells was 0·33 p-equiv. cm^{-2}sec^{-1}.

Fig. 3.—Chloride conductance, g_{Cl} [$= F(\partial\phi_{net}/\partial E)$], as a function of membrane potential, calculated from Figure 2. It was assumed in this calculation that the influx was not a function of potential. At the resting potential ($\simeq$ −150 mV) chloride conductance was 2·6 μmho cm^{-2}.

IV. Discussion

(*a*) *Estimation of Influxes*

MacRobbie (1964, 1966*b*) has shown that by measuring the relative radioactivity in the cytoplasm compared with the whole cell, for short uptake times, it is possible to estimate k_c, the rate constant for chloride exchange in the cytoplasm. It may be shown that

$$\frac{\text{Radioactivity in cytoplasm}}{\text{Radioactivity in cell}} = \frac{1-\exp(-k_c t)}{k_c t}.$$

k_c may be read from families of curves of this function plotted against k_c for various values of t, the uptake time. In turn, $k_c = (\phi_{co}+\phi_{cv})/Q_c$, and consequently the sum of the fluxes out of the cytoplasm can be calculated if Q_c is known. The assumptions inherent in this method are discussed by MacRobbie (1966*b*) and seem

reasonable. Fluxes labelled "influx" in Tables 1 and 2, and in tabulation in Section III(*d*), p. 247, refer to ϕ_{oc} since for short uptake times (several hours) the radioactivity in the whole cell (minus wall) is a linear function of time. These influxes have therefore been calculated from:

$$\phi_{oc} = \frac{\Delta Y}{\Delta t} \cdot \frac{1}{S_o} \cdot \frac{1}{A}.$$

Since S_c (specific activity of the cytoplasm) during these experiments reached less than 1% of S_o, the error due to back-flux of labelled chloride was quite small.

(*b*) *Plasmalemma and Tonoplast Fluxes*

The influx of chloride across the plasmalemma from artificial pond water in light (about 50 cm from two 20-W Daylight fluorescent lamps) varied between about 0·8 and 4 p-equiv. cm^{-2} sec^{-1}. Freshly cut cells, especially shorter, younger cells which had been elongating in culture solution, were found to have higher influxes which decreased to one-half or less of their initial value 1–2 days after excision and after soaking in artificial pond water (Table 1).

Nevertheless, elongation was undetectable in the 24 hr after excision, so that high influxes are not necessarily associated with high rates of growth. Further, in cells which were loaded with Cl^- over periods of 2–4 weeks for the purpose of efflux measurement [Section III(*e*)], the influx averaged as much as 0·5 units even though in natural day and night conditions. Only the light periods would have been effective in promoting a net influx.

The rate constant, k_c, calculated from the proportion of radioactivity found in the cytoplasm after short times of labelling, was about $1\text{–}2 \times 10^{-3}$ sec^{-1} when ϕ_{oc} was 2–3 units. The method of sap sampling gave lower estimates of k_c compared with the perfusion method. During perfusion some radioactivity from the cytoplasm may transfer to the perfusate (either by diffusion or actual loss of cytoplasm) so the lower estimates of k_c are probably more correct. In agreement with MacRobbie (1966*b*) the rate constant was found to be correlated with the influx across the plasmalemma, as shown in Figure 1. This means that $\phi_{co}+\phi_{cv}$ $(=Q_c.k_c)$ is also correlated with ϕ_{oc}, provided Q_c does not vary much between the conditions used to vary k_c and ϕ_{oc} (i.e. light, dark). ϕ_{co} goes *up* rather than down when ϕ_{oc} decreases in the dark, and does not exceed 6–8 units (Hope, Simpson, and Walker 1966). Hence it is apparent that it is the influx across the tonoplast, ϕ_{cv}, which is correlated with ϕ_{oc}. This curious finding remains unexplained. Using a mean value of $Q_c = 90$ n-equiv. cm^{-2} for the chloride content of the cytoplasm it is seen that $\phi_{co}+\phi_{cv}$ must be of the order of 100 p-equiv. cm^{-2} sec^{-1}. Since radioactivity effluxes from cells at a rate corresponding to about 1 p-equiv. cm^{-2} sec^{-1} (Fig. 2), $\phi_{co} \simeq 1$, and $\phi_{cv} \simeq 100$ p-equiv. cm^{-2} sec^{-1}. These values relate to light, or "uninhibited" conditions.

(*c*) *Chloride in the Cytoplasm*

The perfusion technique has been used with *N. flexilis* extensively in connection with ionic studies (Tazawa and Kishimoto 1964). This method is particularly suitable for producing a "cell" for analysis which has the vacuolar ions replaced by an

isosmotic solution containing no K^+, Na^+, or Cl^-. Another method of analysing for cytoplasmic ion content has relied on centrifugation to give layered chloroplasts and cytoplasm with consequent risk of admixture of small vacuoles of vacuolar sap; in her experiments MacRobbie (1966*b*) has eliminated this possibility of contamination. Theoretically, direct *in vivo* measurement of ionic activity may be made using ion-selective glass microelectrodes for Na^+ and K^+, and Ag/AgCl microelectrodes for Cl^-. Coster (1966) has estimated chloride activity in the flowing cytoplasm to be 10 mN in *Chara*, by the last-named method.

TABLE 3

SUMMARY OF PUBLISHED ESTIMATES OF THE CONTENT AND CONCENTRATION OF CHLORIDE IN THE CYTOPLASM OF CHARACEAN CELLS

Plant	Method	Phase	Cl^- Content (n-equiv. cm^{-2})	$[Cl^-]$ (μ-equiv. ml^{-1})	Reference
N. translucens	Cell ends cut and vacuole blown out	Chloroplast layer	215	(240)	MacRobbie (1964)
N. translucens	Centrifugation, electrometric measurement	Clear cytoplasm	—	65	Spanswick and Williams (1964)
N. flexilis	(1) Fast perfusion	Chloroplast layer	—	136	Kishimoto and Tazawa (1965)
	(2) Difference between slow and fast perfusion	Flowing cytoplasm	—	0–40	Kishimoto and Tazawa (1965)
C. australis	Ag/AgCl electrode *in vivo*	Flowing cytoplasm	—	10	Coster (1966)
N. translucens	Centrifugation, Ag/AgCl electrode	Clear cytoplasm	—	90	Hope, Simpson, and Walker (1966)
C. australis	Perfusion, electrometric titration	Chloroplast layer	90	(400)	Present results

Kishimoto and Tazawa (1965), by perfusing both at a slow and fast rate, claim to have produced preparations from which it was possible to estimate the ion contents of the chloroplast layer and flowing cytoplasm, and of the chloroplast layer, respectively; the fast perfusion was designed to remove the liquid cytoplasm along with the vacuolar sap. In the present experiments the estimated Cl^- using either rate of perfusion was the same, which strongly suggests, as do the results of Kishimoto and Tazawa (1965) and Coster (1966), that most of the chloride is in the chloroplast layer. In *C. australis*, 90 n-equiv. Cl^- in a volume of 0·22 μl [both per cm^2—see Section III(*b*)] corresponds to a concentration of 400 mN.

Table 3 presents a summary of the findings to date.

(*d*) *Effects of Inhibitors*

MacRobbie's general thesis (1965, 1966*a*, 1966*b*) is that inward transport of chloride is closely connected with the continuation of electron flow through the known series of reductants and oxidants involved in photosynthesis. The photosynthetic phosphorylation coupled to this electron flow is said to be essential for transport inwards of K^+.

Evidence for these different mechanisms rests heavily on the observed continuance of K^+ influx in far-red light when Cl^- influx is severely limited compared with red light or red light of reduced intensity. Using far-red light conditions, cyclic photophosphorylation is supposed to continue, accounting for K^+ influx.

The differential effect of CCCP, an uncoupler of photosynthetic phosphorylation, lends support to this scheme; it inhibited K^+ influx much more than Cl^- influx at a concentration of 5 μM (MacRobbie 1966*a*, Table 2). Imidazole, another reputed uncoupler, was similar in its effects when used at 10^{-4}M.

In the present experiments somewhat different results have been obtained with CCCP. There appears to be a narrow range of concentration (about 2–3 μM) at which Cl^- influx is significantly inhibited (Table 2), below which it is without effect, and above which it is lethal. Further, the concentration which inhibited Cl^- influx in the light was ineffective in the dark. The effect of CCCP on K^+ influx was not investigated. In *C. australis* there seems less need to postulate that the potassium fluxes are anything but passive. The effect of 10^{-4}M imidazole was stimulatory in *C. australis*, and the same effect was observed for *N. translucens* in two out of three experiments reported by MacRobbie (1966*a*).

Of the effective metabolic "inhibitors" used in this study, phlorizin, which is reported to slow down the rate of photophosphorylation in isolated chloroplasts without much effect on "light-induced swelling" (Nobel 1967), seems to be the most specific in connection with chloride transport. Its effects in chloroplasts are similar to those of oligomycin in mitochondria where ATP production is inhibited while energy-linked processes such as swelling and cation uptake are unaffected or stimulated (for review see Good, Izawa, and Hind 1966).

The action of phlorizin on Cl^- influx in *C. australis* seems to be a direct contradiction of the hypothesis that chloride transport needs only net electron transport without involving ATP or other phosphorylated compounds directly. The other inhibitors used in the present study, or by MacRobbie, affect electron transport either by slowing down net electron flow by eliminating light reaction II (far-red light), by preventing release of electrons from water (DCMU), by interfering with the cytochromes in the redox system (azide), or by diverting electrons from the usual pathway (Paraquat).

In addition, some of the inhibitors, such as puromycin, having a small inhibitory effect both in *Nitella* and *Chara*, may have a more general effect on metabolism than the one assumed (Appelman and Kemp 1966).

The inconclusiveness of the evidence so far presented seems to be due to a failure to make parallel biochemical studies of the penetration and effect of the inhibitors *in vivo*. These studies are proceeding.

(e) *Electrical Conductance Due to Chloride*

The results reported here show that the contribution of chloride ions to the electrical conductance of the membranes increases from a small percentage near the resting potential until at very negative potentials (i.e. for a very hyperpolarized condition) the chloride conductance accounts for most of the total conductance. The electrical conductance first decreased and then increased as the membrane became more hyperpolarized.

Neither of these phenomena are expected from the simpler models of membrane electrical behaviour. For example, Goldman's equation connecting flux with potential indicates a gentle increase in flux as the p.d. gets further away from the equilibrium p.d. for the ion in question, and a *decrease* in total conductance (increase in resistance) on the hyperpolarizing side of the resting p.d. A decrease in conductance is observed for hyperpolarization $\simeq 100$ mV (Figs. 6 and 7, Coster 1965) but thereafter conductance increases dramatically at a particular membrane potential. This sudden increase in conductance has been termed "punch-through" (Coster 1965) and occurs in *Chara* with about -170 mV bias, i.e. for a plasmalemma p.d. of about -300 mV.

The present results show that large effluxes of chloride ions occur in the region of the punch-through potential, which lends support to the suggestion that the plasmalemma, or part of it, may be a double, fixed-charge membrane with a positively charged lattice or pore system forming an abrupt junction with a negatively charged lattice or pore. In such a system at the junction of the two fixed-charge regions there is a double space-charge region which is almost completely depleted of diffusible ions. It can be shown that the width of this depletion layer increases with increasing hyperpolarization and that punch-through occurs when the space-charge regions extend to the membrane boundary (Coster 1965).

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