Factors Affecting the Fluxes of Potassium and Chloride Ions in *Nitella translucens*

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ABSTRACT A more complete study of ionic concentrations and fluxes in the giant internodal cells of *Nitella translucens* has been made. The vacuolar concentrations were 76 mM K and 170 mM Cl. The content of the chloroplast layer was 135 mμmoles K/cm^2 and 215 mμmoles Cl/cm^2; in a layer 9 μ thick these correspond to concentrations of 150 mM K and 240 mM Cl. Such a high level of chloride requires active transport of chloride into the cytoplasm, either at the plasmalemma or at the membranes bounding the cytoplasmic particles; it cannot be achieved by active transport of chloride only at the tonoplast. With concentrations of 0.1 mM K and 1.3 mM Cl outside, the fluxes into the cytoplasm had mean values of 1.0 to 1.4 $\mu\mu$moles K/cm^{2}sec. and 2.1 to 2.8 $\mu\mu$moles Cl/cm^{2}sec.; the corresponding fluxes from the cytoplasm to the vacuole were about 110 $\mu\mu$moles K/cm^{2}sec. and 175 $\mu\mu$moles Cl/cm^{2}sec. The transfer of both potassium and chloride to the vacuole under different conditions appeared to be correlated with the uptake of chloride into the cytoplasm. It is suggested that two separate processes are involved in the active accumulation of salts in the vacuole—an active uptake of chloride in the cytoplasm and a subsequent transfer of salt to the vacuole. It may be that the second process involves the formation of small vesicles in the cytoplasm and their subsequent discharge into the central vacuole.

It has previously been shown that the normal ionic state of the giant internodal cells of the fresh water characean *Nitella translucens* is maintained by active transport of both cations and anions; the high potassium/sodium ratio is maintained by an active influx of potassium and an active efflux of sodium at the outer membrane, the plasmalemma, whereas the high total salt concentration arises by an active uptake of chloride (MacRobbie, 1962). The site of the chloride transport was not established, although it was argued that osmotic equilibrium between cytoplasm and vacuole required the chloride transport to be at the tonoplast, the inner protoplasmic membrane.

This paper reports work on the chloride concentrations and fluxes in

Nitella translucens, from which it appears that the osmotic argument is unsound; the chloride content of the chloroplast layer is very high and is not compatible with a passive distribution across the plasmalemma. It appears therefore that there is active transport of chloride at the plasmalemma, and that, although this is independent of the cation transport mechanism, the two are located at the same membrane.

It was shown previously that the tonoplast is a much more permeable structure than the plasmalemma, but the tonoplast fluxes were not measured. The present paper reports fluxes of potassium and of chloride at the tonoplast, and confirms that these are very high.

METHODS

Methods used were similar to those described previously (MacRobbie, 1962). Experiments were done on single internodal cells of *Nitella translucens*, which were 3 to

TABLE I

ION CONCENTRATIONS IN THE SOLUTIONS USED

Solution		Na	K	Ca	Cl	SO_4
		mM	*mM*	*mM*	*mM*	*mM*
Artificial pond water	A	1.0	0.1	0.1	1.3	—
	B	3.0	0.1	0.1	1.3	1.0
	C	7.0	0.1	0.1	1.3	3.0
Low chloride	LA	1.0	0.1	0.1	0.3	0.5

8 cm long and 600 to 1100 μ in diameter. Cells remained healthy in the laboratory in artificial pond water for some months. Only turgid cells showing rapid protoplasmic streaming were used.

The chemical composition of the solutions used is given in Table I. These solutions were labelled with the isotopes K^{42} or Cl^{36}, as described before.

Cells were soaked in radioactive solution for 12 to 60 hours, in a tank illuminated by two 40 watt "warm white" fluorescent tubes, at a temperature maintained within ± 0.5°C at 10, 20, or 25°C. Additional aeration was not provided as it had been shown to have no effect on the results.

Sample Analyses

The chemical content and radioactivity were determined separately for the chloroplast layer and for the vacuolar sap. Sodium and potassium were determined in an EEL flame photometer, and chloride by electrometric titration (Ramsay, Brown, and Croghan, 1955).

K^{42} was determined by liquid counting (20th Century M6 tubes), and Cl^{36} was counted on planchettes under an end-window Geiger tube (Mullard MX 123). Low chloride samples were counted in an anticoincidence arrangement in which the background was only 3 counts/minute instead of the normal end-window back-

ground of 12 counts/minute. Standards were counted under identical conditions for comparison.

Separation of Samples

The cell was removed from the solution and blotted to remove adhering liquid. One end of the cell was cut and the open end threaded onto a fine pipette; the other end was then cut and the fluid contents blown out into liquid paraffin, while the chloroplast layer remained attached to the cell wall. The nature of this layer will be discussed later.

Samples of clear sap obtained in this way were weighed on a torsion microbalance (maximum error 0.03 mg), and were then diluted with distilled water for liquid counting and flame photometry, or titrated for chloride, or counted on planchettes.

In potassium experiments a length of the sleeve of chloroplast layer plus wall was put into 0.5 ml of N HNO_3 to extract the ions; this was then diluted with distilled water for liquid counting and flame photometry. In chloride experiments a length of the sleeve was cut up and put into a small polythene specimen tube (5 mm in diameter, 1 cm in height), and a drop of N HNO_3 added; this was then titrated for chloride. The silver chloride precipitate was dissolved in ammonia, and the solution transferred quantitatively to a 25 mm planchette; the sample was then made alkaline by an excess of sodium hydroxide in alcohol to prevent loss of chloride during drying.

The method provides a sample of chloroplast layer plus cell wall and it is necessary to consider the contribution of the ions in the wall. Chloride is present only in the water-free space of the wall (Dainty and Hope, 1959), and hence it represents a very small fraction of the total in the sample. Potassium is present in much higher amount, mainly in the Donnan phase in the wall, and must therefore be estimated separately and subtracted from the total. It was assumed from previous work that the total potassium in the cell wall was equal to the amount of K^{42} taken up in a 10 minute period in radioactive solution and washed out in a subsequent 20 minute period into a 10 ml portion of inactive solution which was then counted; these times were chosen in the light of the time course of uptake shown in the previous paper (MacRobbie, 1962). The contribution of potassium in the wall to the total was about 10 to 25 per cent of the radioactivity but only about 2 to 3 per cent of the total potassium.

From these analyses the specific activity of the ion concerned was estimated separately in the chloroplast layer and in the cell sap after varying periods of uptake. From the specific activities estimates of the fluxes at the two membranes were made.

Estimation of Fluxes

The cell is considered as two intracellular compartments—the cytoplasm and the vacuole—in series with one another, and it is assumed that the specific activity in the chloroplast layer is a fair estimate of that in the cytoplasm as a whole.

The specific activities in the cytoplasm and in the vacuole are given by the equations:

$$Q_C \frac{ds_c}{dt} = A\,[M_{OC} + M_{VC}\,s_v - (M_{CO} + M_{CV})\,s_c] \qquad (1)$$

$$C_V V_V \frac{ds_v}{dt} = A [M_{CV} s_c - M_{VC} s_v] \quad (2)$$

where M_{OC} = flux from outside to cytoplasm (amount per unit area per unit time)
M_{CO} = flux from cytoplasm to outside
M_{CV} = flux from cytoplasm to vacuole
M_{VC} = flux from vacuole to cytoplasm
s_c = cytoplasmic specific activity as a fraction of that outside
s_v = vacuolar specific activity as a fraction of that outside
Q_C = total amount of ion in the cytoplasm
A = area of cell
C_V = vacuolar concentration of ion
V_V = vacuolar volume

The flux at the tonoplast is much higher than that at the plasmalemma (MacRobbie, 1962, and the present paper), and therefore most of the activity entering the cell goes through into the vacuole, and the cytoplasmic specific activity remains low. Under these conditions the cytoplasmic activity will rise to a quasi-steady level in which its rate of rise is very small compared with the tracer flux through it, and it will subsequently rise only very slowly as the specific activity rises in the very large vacuole. The cytoplasmic specific activity is then given by

$$s_c = \frac{M_{OC} + M_{VC} s_v}{M_{CO} + M_{CV}} \quad (3)$$

and the rate of rise of specific activity in the vacuole is given by equation (2). We may call the apparent influx to the vacuole (measured by the rate of rise of specific activity in the vacuole) M_1', given by

$$M_1' = \frac{V_V}{A} C_V \frac{ds_v}{dt}$$

The true fluxes at the two membranes are then related to the measurable quantities M_1', s_c, and s_v by the equations:

$$M_1' = M_{CV} s_c - M_{VC} s_v \quad (4)$$

and from (3)

$$M_{OC} - M_{CO} s_c = M_{CV} s_c - M_{VC} s_v = M_1' \quad (5)$$

In the normal solution the cells are in a steady state, and we may write $M_{CV} = M_{VC} = M_2$, the flux in either direction at the tonoplast, and $M_{OC} = M_{CO} = M_1$, the flux in either direction at the plasmalemma. Thus for cells in a steady state we have

$$M_2 = M_1'/(s_c - s_v) \text{ and } M_1 = M_1'/(1 - s_c)$$

These calculations rest on the assumption of flux equilibrium, which is valid for the

standard bathing solution, but needs further examination under other conditions. The ion content of the cytoplasmic layer is much less than the amount crossing the tonoplast per hour, and the half-time for chloride exchange between chloroplast layer and vacuole must be of the order of 20 minutes or less; that for potassium is longer but still short compared with the experimental times. This would seem to imply that a new flux equilibrium between cytoplasm and vacuole is set up rapidly if conditions change, and that it is therefore valid to put $M_{CV} = M_{VC} = M_2$ except in the initial period of adjustment. It has therefore been assumed that the equation above can be used to calculate M_2 in experiments of 12 to 60 hours' uptake, in conditions other than the standard ones. As s_c is generally less than 10 per cent of the specific activity outside, the correction to M_1' to give the true influx is always small, and the assumption that $M_{OC} = M_{CO}$ affects the calculation of M_1 only very little; it has therefore been assumed that it is valid to use both the above equations.

Justification for These Assumptions

The assumptions of this treatment which must be verified by the results may be restated as follows:—

1. The cell may be considered as two intracellular compartments, the cytoplasm and the vacuole, in series with one another
2. The relative fluxes at the plasmalemma and at the tonoplast are such that the cytoplasmic specific activity reaches a quasi-steady level (much less than that outside), and then rises only very slowly as the vacuolar specific activity rises; during this time an almost constant difference of specific activity is maintained between the two

$$\left[s_c - s_v = \frac{M_1}{M_1 + M_2}(1 - s_v)\right]$$

3. The specific activity of the chloroplast layer is a fair estimate of the specific activity of the cytoplasm as a whole.

It is convenient to discuss the justification for these assumptions at this stage, anticipating the results which follow.

1. Diamond and Solomon (1959) found that the rise of specific activity in the vacuole of *Nitella axillaris* showed the lag period characteristic of a compartment separated from the medium by another compartment in which the initial radioactivity was zero, and in which the subsequent rise was slow. In preliminary experiments a similar lag period was found in *Nitella translucens*; furthermore the length of this lag was consistent with the values found for the cytoplasmic ion content and the fluxes at the tonoplast.

During the initial period before the quasi-steady level is reached, and when s_v is still negligible, the cytoplasmic specific activity at time t is given by

$$s_c = \frac{M_1}{M_1 + M_2}(1 - e^{-kt}) = s_{cs}(1 - e^{-kt})$$

where k, the rate constant for exchange in the cytoplasm, is equal to

$$(M_1 + M_2)\, A/Q_C$$

The rate of rise of activity in the vacuole at time t is given by

$$\frac{V_V\, C_V}{A}\frac{ds_v}{dt} = M_2\, s_c = M_2\, s_{cs}(1 - e^{-kt})$$

The total entry to the vacuole in the period up to time t is obtained by integrating this equation, and the apparent influx to the vacuole during this period is then obtained by dividing this total by the time.

Thus the apparent influx in the time t is equal to

$$M_2\, s_{cs} - M_2\, s_{cs}\frac{(1 - e^{-kt})}{kt}$$

TABLE II

CALCULATION OF RATE CONSTANT FOR CYTOPLASMIC EXCHANGE FROM THE LAG PERIOD IN VACUOLAR UPTAKE: POTASSIUM

Time of uptake t	Mean influx	No. of cells	Influx as fraction	Fractional decrement $\frac{(1 - e^{-kt})}{kt}$	kt	k (hr^{-1})
hrs.	*μμmoles/cm² sec*					
16.0	1.00	11	1.00	0.00	—	—
7.4	0.78	3	0.78	0.22	4.5	0.61
4.67	0.75	7	0.75	0.25	3.9	0.83
2.2	0.52	5	0.52	0.48	1.7	0.77
1.6	0.44	1	0.44	0.56	1.3	0.81
1.2	0.25	4	0.25	0.75	0.6	0.50

This falls short of its final value at $t \rightarrow \infty$ by the fraction

$$\frac{1 - e^{-kt}}{kt}$$

of its final value, and hence from the estimates of the apparent influx at short times a value for k may be calculated. In Table II are given figures for the apparent influx of potassium to the vacuole (M_1') for various periods of uptake. It is clear that the full influx does not develop immediately but that there is a lag period. The values of k calculated from 20 cells at five times of uptake are shown, from which it appears that the rate constant for cytoplasmic potassium exchange is about 0.7 hr.$^{-1}$. The mean values for the potassium content of the chloroplast layer isolated from the 1961 cells were in the range 137 to 188 mμmoles/cm² and the sum of the fluxes ($M_1 + M_2$) for such material (see Table VIII) had a mean value of 33 μμmoles/cm.²sec.; together these figures would give a value of 0.63 to 0.85 hr.$^{-1}$ for the rate constant. Thus the initial lag period in the process of transfer of activity to the vacuole is con-

sistent with the figures for potassium content of the cytoplasm and for the tonoplast flux found later.

2. The specific activities of potassium in the chloroplast layer and in the vacuole of a number of cells were determined after times of uptake from 1 to 29 hours. During this period the specific activity in the vacuole rose from 0.1 to 7.2 per cent of that in the medium, and the specific activity in the chloroplast layer rose from 1.8 to 11.0 per cent. It is clear from Fig. 1, in which the difference ($s_c - s_v$) is plotted against s_v, that this difference remained approximately constant as s_v rose over this range. Thus it appears that the flux at the tonoplast is so much higher than that at the plasmalemma that the cytoplasmic radioactivity does reach a quasi-steady level, after which it rises only slowly as the vacuole fills up.

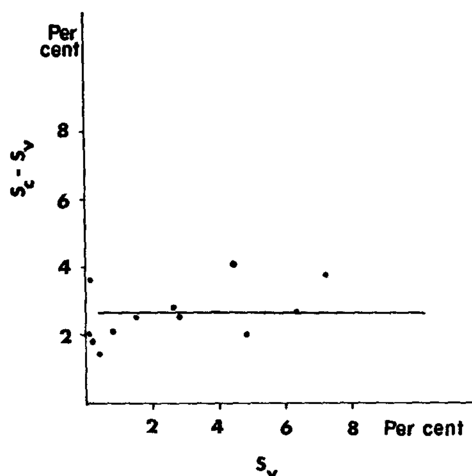

Figure 1. The difference in specific activities in the cytoplasm and in the vacuole (s_c-s_v) plotted against the vacuolar specific activity (s_v), during the uptake of potassium. Each specific activity is expressed as a percentage of that outside.

3. The main justification for the assumption that the specific activity measured in the chloroplast layer is a fair estimate of that in the cytoplasm as a whole, comes from experiments in which the radioactive solution was applied to one end only of the cell, and the rate of transfer of activity to the other end was measured. The cell was placed in a vaseline/wax seal in a groove in a perspex partition separating two chambers; when active solution was placed in one of these chambers labelled ions were taken up by the part of the cell in that chamber, and carried to the other end of the cell by cytoplasmic streaming. The specific activity remained low enough in the cell for any subsequent loss of tracer to the medium to be neglected.

In preliminary experiments with potassium it was found that in such uptake experiments the total amount of activity in both ends (determined by direct counting of each end of the cell) rose linearly with time. In typical experiments the rate of rise of activity in the "inactive" end was about 50 per cent of the rate of increase in the "active" end of the cell. As the flowing cytoplasm and the stationary layer are of comparable volumes this suggested that there could be no marked difference in specific activity between them.

In later experiments, using both radioactive bromide and chloride, the activities in the vacuole and in the chloroplast layer in each end were determined separately.

In Table III the ratios

$$\frac{\text{Activity in given phase in the active end of the cell}}{\text{Activity in given phase in the inactive end}}$$

are quoted for the chloroplast layer and for the vacuole.

It seems reasonable to assume that the ratio for the vacuole reflects the ratio of the specific activities, in the two ends, of the cytoplasmic phase from which the vacuole receives its activity; there is reasonable agreement between the vacuolar ratio and

TABLE III

HALF-LABELLING EXPERIMENTS: CHLORIDE AND BROMIDE

Ion	Time of uptake	$\frac{C^*_V \text{ in active end}}{C^*_V \text{ in inactive end}}$ (Vacuole)	$\frac{C^*_C \text{ in active end}}{C^*_C \text{ in inactive end}}$ (Chloroplast layer)
	hrs.		
Cl	3.8	1.9	1.8
Cl	4.9	34	26
Cl	5.7	1.3	1.5
Cl	6.0	2.1	3.2
Cl	16.25	2.3	2.0
Cl	17.25	1.3	1.1
Cl	17.5	1.2	1.8
Cl	17.8	1.1	1.2
Cl	3.2	22	18
Cl	3.6	1.8	1.7
Cl	3.9	6.3	13
Cl	4.0	1.1	1.1
Br	1.7	1.9	2.2
Br	1.9	1.5	1.55

that in the chloroplast layer isolated, suggesting that this layer gives a fair estimate of the specific activity in the phase supplying the vacuole. It therefore seems valid to assume that the specific activity measured in the chloroplast layer may be used to calculate meaningful fluxes.

It is much more difficult to isolate these fractions from two halves of a cell quickly and cleanly than to obtain single samples of these fractions from each cell; in view of this, the few discrepant ratios in Table III are not thought to invalidate the conclusion drawn. (It is assumed that the 3 cells in which both ratios are very high are cells whose streaming did not recover its normal fast rate with the cell in the partition chamber, and in which, therefore, the rate of transfer to the inactive end was very low.)

Nature of the Cytoplasmic Fraction

The *Nitella* cell may be regarded as a cylinder, in which the cytoplasm forms a thin layer lining the cell wall. The layer immediately inside the wall is gel (approximately 6 μ thick), in which the chloroplasts are embedded in a regular spiral (helical) ar-

rangement; inside that is the layer of flowing cytoplasm moving along the spiral course. The total cytoplasmic thickness is about 10 to 15 μ. When the fluid contents are blown out a layer of liquid must remain wetting the gel layer left sticking to the wall. It is believed that the fluid adhering is flowing cytoplasm and not sap, for reasons given below. The question of possible sap contamination is important in the calculation of differences of specific activity between cytoplasm and vacuole, and must be considered in some detail.

(a) THICKNESS OF LAYER

The sleeve of wall plus chloroplast layer was weighed (to 0.03 mg) and the time course of water loss by evaporation was followed; the first reading could be made within 1 minute of removal from the solution, and the weight was determined at 1 minute intervals for 5 to 10 minutes. The wet weight of the sample was determined by extrapolation of the graph to zero time. The adhering chloroplast layer was then removed by sucking distilled water up and down the sleeve, and squeezing it with forceps. The clear cell wall was then blotted and its wet weight was determined in a similar manner. By substraction the wet weight of the chloroplast layer removed was obtained. The mean value for 8 cells, of the wet weight of the chloroplast layer was 0.92 $\pm$ 0.09 mg/cm^2 (mean $\pm$ SEM); if the density is taken as 1 this would give a thickness of 9 μ, and with a density greater than 1 the thickness would be correspondingly less. In any case the thickness is considerably less than the total thickness of the cytoplasm, and it seems very unlikely that it includes any sap adhering to the chloroplasts. It appears rather that the contaminating fluid left, such as there is, is flowing cytoplasm; this should not affect the determination of specific activity in the cytoplasm but only the total amount of ion in the sample.

(b) VARIABILITY OF SAMPLE OBTAINED

In most cells very few chloroplasts are dislodged by the blowing-out of the sap; if the sap blown into paraffin appeared green and contained a significant number of chloroplasts, the sample was discarded. The cut end of the cell was drained on paper tissue and any fluid remaining was blown out; this procedure usually produced a green sample (not included in the sap sample) and if the cell wall had been bent at all some fluid remained in the bend. The fluid remaining appeared to be densely granular and is thought to be flowing cytoplasm and not vacuolar sap. These two factors, some loss of chloroplasts and differences in the amount of flowing cytoplasm lining the layer, mean that the sample obtained is a variable proportion of the cytoplasm of the cell, and hence there is a wide spread in the ion content (in mμmoles/cm^2) of the layer obtained. It was found, however, that even when the total amounts in two lengths of the sleeve from the same cell were very different, the specific activities of the two samples were consistent. This was true even for very short times of uptake, when the vacuolar specific activity was only 10 to 20 per cent of that in the cytoplasm. Thus the specific activity of the extra ion content in the higher sample was that of the cytoplasm rather than that of the vacuole, arguing against contamination of the layer with vacuolar sap.

Attempts were made to detect any sap contamination by introducing a dye or a

fluorescent dye into the pipette before blowing out the sap. After blowing this solution through there did not appear to be coloured or fluorescent solution left on the chloroplast layer, except in the short lengths at the two ends which had been damaged in cutting; these lengths were not in any case included in the sample.

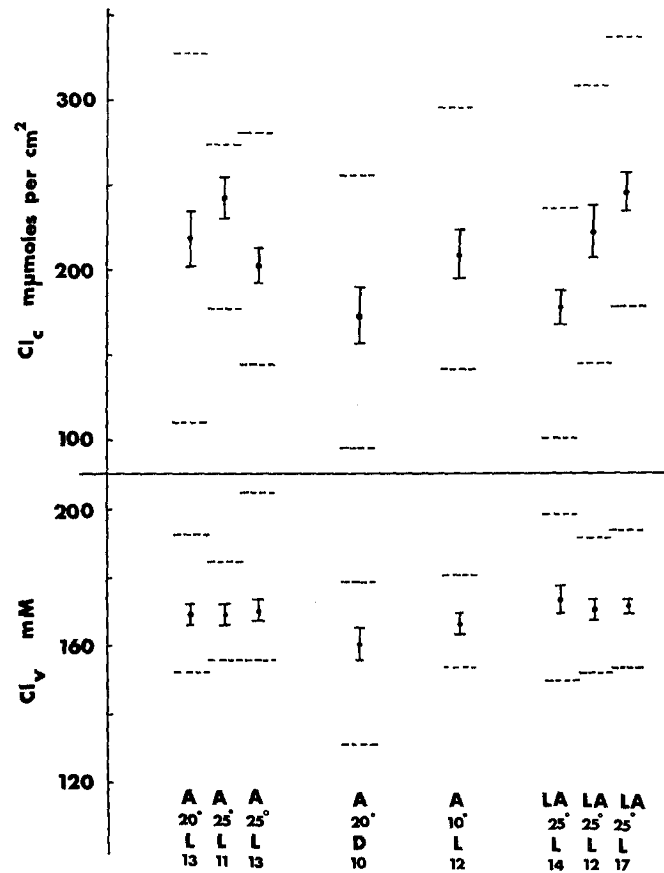

FIGURE 2. Concentration of chloride in the vacuole Cl_V(mM), and chloride content of the cytoplasmic layer Cl_C (mμmoles/cm²), in experiments under different conditions. The mean, standard error of the mean, and range, are shown in each case; the figures below define the conditions and specify the solution used, the temperature, whether light or dark, and the number of cells measured. Solution A has Cl_0 = 1.3 mM, solution LA has Cl_0 = 0.3 mM; both solutions have the same cation composition.

RESULTS

Chloride Content of the Samples

The pooled results of three experiments under "normal" conditions (20 or 25°C, light, outside solution A with Cl_0 = 1.3 mM) are given below. Results will be quoted in the form: mean ± standard error of mean (number of results on which mean is based).

Chloride concentration in the vacuole, Cl_V (mM): 170 ± 2 (36)

Chloride content of the chloroplast layer, Cl_C (mμmoles/cm²): 215 ± 7 (36)

If the adhering layer is about 9 μ thick this would correspond to a chloride concentration of about 240 mM, and there seems to be little doubt that the concentration is higher in the chloroplast layer than in the sap, even if its precise value is not accurately known.

The spread of values of Cl_C within the same experiment, and between different experiments at different times of year, was much wider than that in Cl_V, but it has already been explained that the method provides a sample of a rather variable proportion of the total cytoplasm.

Experiments were done under different conditions of light, temperature,

TABLE IV
POTASSIUM CONTENT OF THE VACUOLAR SAP AND OF THE CHLOROPLAST LAYER

Vacuole		Chloroplast layer		
K_V	Range	K_C	Range	Mean concentration as a layer of thickness 9μ
mM		*mμ moles/cm²*		*mM*
76±1 (67)	52–102	135±4 (69)	71–230	150±5

and external chloride concentration, and the results are collected in Fig. 2; this shows, for each experiment, the mean and its standard error, the range of values found, and the number of cells measured under the conditions specified. All the material was 1962 growth, the final experiment in March, 1963, being on material which had been stored for 5 months in the laboratory but which appeared to be still in a healthy state.

It should be noted that the vacuolar chloride in this material is significantly higher than that of the 1960–61 material, which was only 151 mM. The pond from which the material was collected showed much more vigorous growth of *Nitella* in 1962, and it will be seen later that the influx of chloride was three to four times higher in this material than in the previous sample. There seems to be no doubt that the difference is genuine and that conditions in the pond were, for reasons unknown, more favourable for growth in 1962.

Potassium Content of the Samples

The pooled results for the potassium content of the chloroplast layer and for the potassium concentration of the cell sap are given in Table IV. The amount in the chloroplast layer would correspond to a concentration of 150 mM in a layer 9 μ thick.

Chloride Fluxes: Flux at the Plasmalemma: M_1

The mean values for the influx of chloride at the plasmalemma under different conditions are collected in Table V. It should be noted that these fluxes are

TABLE V

INFLUX OF CHLORIDE AT THE PLASMALEMMA: M_1

Solution	Conditions	Cl_0	Time	Mean Influx, M_1	Range of M_1
		mM			
A	Light, 20°,	1.3	June	2.8±0.2 (13)	1.5–4.2
A	Light, 25°,	1.3	Aug.	2.1±0.2 (11)	1.3–3.7
A	Light, 25°,	1.3	March	2.6±0.2 (13)	1.4–3.8
A	Dark, 20°,	1.3	June	0.18±0.04 (10)	0.08–0.39
A	Light, 10°,	1.3	Dec.	1.5±0.1 (12)	1.2–2.1
LA	Light, 25°,	0.3	Aug.	1.7±0.15 (10)	1.1–2.9
LA	Light, 25°,	0.3	Oct.	1.3±0.2 (12)	0.6–2.1
LA	Light, 25°,	0.3	Dec.	1.4±0.1 (11)	1.0–2.3
LA	Light, 25°,	0.3	March	1.75±0.1 (11)	1.2–2.1

All fluxes in $\mu\mu$moles/cm² sec.

TABLE VI

APPARENT INFLUX OF CHLORIDE TO THE CELL: M_1'

Solution	Conditions	Mean M_1'	Range
A	Light	3.2±0.1 (12)	2.7–3.6
B: A+1 mM Na_2SO_4	Light	4.1±0.15 (9)	3.5–5.0
C: A+3 mM Na_2SO_4	Light	4.1±0.15 (11)	3.0–5.0
B	Dark	0.23±0.04 (12)	0.08–0.43
B+5 × 10^{-6} M DCMU	Light	0.25±0.05 (9)	0.16–0.56
Earlier experiments:	April–May:	all at 20°C	

All fluxes in $\mu\mu$moles/cm² sec.

much higher than those measured in the previous work, where the mean chloride influx was only 0.85 $\mu\mu$moles/cm² sec. (light, 20°C, Cl_0 = 1.3 mM).

From these results it appears that the influx of chloride at the plasmalemma is reduced, but not proportionately, by lowering the external chloride concentration, has a Q_{10} approaching 2, and is very markedly reduced in the dark, by a factor of about 16.

The results of some earlier experiments in which only the apparent influx to the cell M_1', was measured, are shown in Table VI. For the higher values

the specific activity in the cytoplasm was about 7 to 10 per cent and M_1' is therefore less than the true influx M_1 by this amount; for the two low values the underestimate can only be by 1 to 2 per cent.

The table shows that the influx is reduced by the photosynthetic inhibitor dichlorophenyl-dimethylurea (DCMU) at a concentration of 5×10^{-6} M, to a value very close to that in the dark. It also shows that the chloride influx is increased somewhat by the presence of extra sodium in the medium, added as sodium sulphate.

TABLE VII
FLUX OF CHLORIDE AT THE TONOPLAST: M_2

Solution	Conditions	Cl_0	Time	Mean M_2	Range of M_2
		mM			
A	Light, 20°,	1.3	June	169±23 (13)	66–318
A	Light, 25°,	1.3	Aug.	182±30 (11)	75–405
A	Dark, 20°,	1.3	June	32±10 (10)	5–55
A	Light, 10°,	1.3	Dec.	80±15 (12)	17–178
LA	Light, 25°,	0.3	Oct.	54±10 (11)	11–97
LA	Light, 25°,	0.3	Dec.	116±16 (11)	53–205

All fluxes in $\mu\mu$moles/cm² sec.

Chloride Flux at the Tonoplast: M_2

The values calculated for the chloride flux at the tonoplast are given in Table VII, and some comment may be made at this point on the very wide range of values found. A part of this may come from the decreasing accuracy of the estimate of M_2 as its value increases. For example, if M_2 is 180 $\mu\mu$moles/cm² sec. and M_1' is 2.7 $\mu\mu$moles/cm² sec., then the difference in specific activities in the cytoplasm and vacuole ($s_c - s_v$) is only 1.5 per cent of the specific activity outside; in general it is easy to reach a high degree of accuracy in the estimate of s_v from a large sap sample, but the size and activity of the chloroplast sample limit the accuracy obtainable for s_c in any reasonable counting period. Thus in this example if s_c is about 5 per cent of s_o, and is determined with an accuracy of 2 to 5 per cent of its value, then the value of ($s_c - s_v$), and hence M_2, will have uncertainties of 7 to 16 per cent of their values. Although low values of M_2 can be determined to 2 to 5 per cent the errors in the estimation of higher values become progressively greater. But this does not explain the very wide range found, and it appears that this spread is to a large extent calculable. This will be dealt with in a later paper.

It appears from Table VII that the tonoplast flux is also reduced by the factors affecting the flux at the plasmalemma, light, temperature, and external

chloride concentration. The mean values of the tonoplast flux M_2 in different experiments are plotted against the corresponding values of M_1, in Fig. 3. (In the graph one of the experiments of the table (LA, Oct.) has been separated into two groups of cells, one having a much longer period in the low chloride solution; this explains why there is apparently an extra point.) It is clear from this graph that there is a correlation between the chloride fluxes at the two membranes.

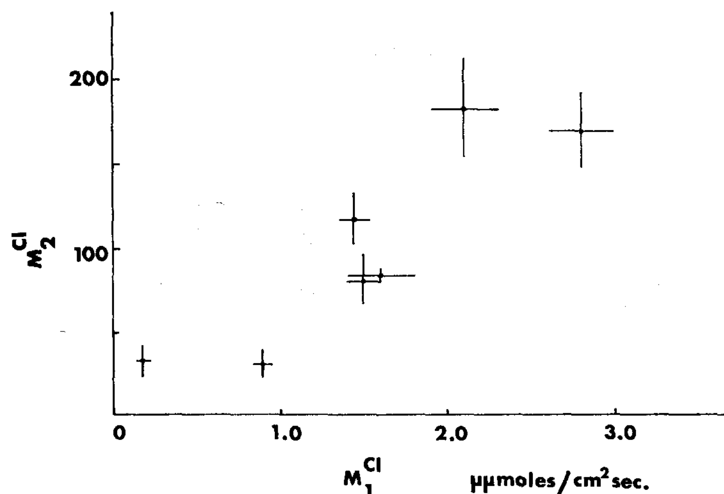

FIGURE 3. Flux of chloride from cytoplasm to vacuole M_2^{Cl}, plotted against the influx of chloride from the external solution to the cytoplasm M_1^{Cl}. In order of increasing M_1^{Cl}, the points represent the following conditions:
Dark, 20°C, Cl_0 1.3 mM
Light, 25°C, Cl_0 0.3 mM (2)
Light, 10°C, Cl_0 1.3 mM
Light, 25°C, Cl_0 0.3 mM
Light, 25°C, Cl_0 1.3 mM
Light, 20°C, Cl_0 1.3 mM.

Potassium Fluxes

Potassium fluxes at both membranes were measured in experiments under the same conditions; the results are shown in Table VIII.

Chloride fluxes were measured in the same experiments, on the same cells, and it is clear from Table VIII that the reduction of the influx of chloride at the plasmalemma in solutions of low chloride is accompanied not only by a reduction in chloride flux at the tonoplast, but also by a reduction in potassium flux at the tonoplast. The mean values of the fluxes in 1961 material are also shown, although in this case the potassium and chloride fluxes were determined, not on the same cells, but on comparable groups of cells.

The potassium influx at the plasmalemma is reduced in the solution containing only sulphate, but is not significantly affected by the reduction of Cl_0 from 1.3 to 0.3 mM. This change decreases the transfer of potassium from the cytoplasm to the vacuole although there is no corresponding decrease in the influx of potassium at the plasmalemma.

DISCUSSION

The first conclusion that may be drawn from these results is that it is not possible to explain them in terms of active transport of chloride at the tonoplast only; it is necessary to assume an active transport of chloride into the cytoplasm. The electric potential of the cytoplasm is negative with respect to the outside solution, by about 140 mv. This means that the concentration of chloride in the cytoplasm in passive equilibrium with the external solution could be only about 0.5 μM. Thus a chloride content of the chloroplast layer of 215 mμmoles/cm^2, or about 240 mM, must be the result of active uptake rather than passive diffusion. Both anions and cations in this layer are in higher concentrations than in the sap, but the two phases must nevertheless be in osmotic equilibrium. The major part of the osmotic pressure of the sap is contributed by sodium and potassium chlorides, so that, even ignoring any contribution of organic molecules to the osmotic pressure of the cytoplasm, the previous assumption, that there is in the cytoplasm no structure capable of supporting a hydrostatic pressure difference, is seen to be invalid. It seems most reasonable to suppose that the chloroplasts themselves have some structural rigidity and are capable of withstanding the required pressure

TABLE VIII

POTASSIUM FLUX AT THE PLASMALEMMA (M_1^K) AND AT THE TONOPLAST (M_2^K); WITH THE CHLORIDE FLUX AT THE PLASMALEMMA (M_1^{Cl})

Cl_0	Time	M_1^K		M_2^K		M_1^{Cl}	No. of cells
		Mean	Range	Mean	Range	Mean	
mM							
1.3	July	0.99±0.07	0.65–1.60	115±30	24–298	2.1±0.1	16
1.3	Aug.	1.4±0.1	0.8–2.1	107±27	26–265	2.4±0.15	11
0.3	Aug.	0.96±0.06	0.62–1.26	45±14	7–110	1.7±0.15	10
0.3	Dec.	1.12±0.12	0.52–2.05	28±6	3–82	1.4±0.1	17
0	Nov.	0.44±0.03	0.30–0.67	16±4	5–48	0	10
*1.3	1961 cells	1.1±0.1	0.4 –1.8	33±2.5	11–57	(0.87±0.06)	25

Fluxes in $\mu\mu$moles/cm^2 sec.

* Chloride fluxes were not measured on the same cells, but on a comparable group of the same material. Other entries in the table refer to measurements of potassium and chloride fluxes on the same cells.

difference. Certainly any damage to the cell, or to the chloroplasts, produces a very marked shrinkage of the chloroplasts, suggesting that they are normally maintained in a swollen state by processes very sensitive to injury.

This argument suggests active chloride movement at the chloroplast membranes and at either the tonoplast or the plasmalemma. Thus there may be either (*a*) an active uptake initially at the plasmalemma, giving high chloride throughout the cell, with further accumulation in the chloroplasts or (*b*) passive distribution of chloride at the plasmalemma with very low chloride in the cytoplasm outside the chloroplasts, but with accumulation into the chloroplasts and into the vacuole. Spanswick and Williams (personal communication) have reported high chloride concentrations (65 $\pm$ 3 (20) mM) in the flowing cytoplasm, which suggests that the first alternative is correct—an active chloride transport at the plasmalemma maintaining a high chloride concentration throughout the cell, with further accumulation within the chloroplasts. The possibility remains, however, that the bulk of the chloride in the flowing cytoplasm is in the mitochondria, and the bulk of the chloride in the chloroplast layer is inside the chloroplasts, leaving only very low concentrations of chloride in the extraparticulate fractions of either phase.

In any of the conditions quoted the ion fluxes at the tonoplast were much greater than those at the plasmalemma; this shows that the plasmalemma is a much more significant barrier to ion movement than the tonoplast. However, the dependence of the ion fluxes at the tonoplast on temperature, light, and external chloride concentration, suggests that these fluxes are not the result of simple passive diffusion.

It is thought that the correlation between the rate of active uptake of chloride into the cytoplasm and the rate of transfer of both potassium and chloride from the cytoplasm to the vacuole is a real one, and cannot be due to any systematic error in sampling or analysing the cytoplasmic fraction. The correlation is such that, of two cells containing the same amount of activity in the vacuole after appropriate periods of uptake, the cell of higher total influx (*i.e.* entry per unit time) has *less* activity in the cytoplasm. Thus a cell with a high influx retains less activity in the cytoplasm and passes a relatively higher proportion into the vacuole, and it is the cells of very low influx which retain the largest amount in the cytoplasm. (This statement implies that fractional changes in M_2 are greater than the corresponding fractional changes in M_1; this is true of the individual cells in any of the groups from which the quoted means are calculated, and is also true of the general relationship shown by the six means in Fig. 3 which refer to different conditions in light.)

Furthermore, it would seem that any apparent correlation arising as an artifact would show up in both potassium and chloride experiments. There was no correlation between M_1^K and M_2^K, either between the means of different

experiments under different conditions, or between different cells having different influxes in any one experiment. It is difficult to imagine any systematic error which would produce a spurious relation between $(s_c - s_v)^{Cl}$ and M_1^{Cl}, without producing an equally spurious relation between $(s_c - s_v)^K$ and M_1^K. Also, the relation between $(s_c - s_v)^K$ and M_1^{Cl} seems quite impossible to explain in terms of systematic error in separation or analyses of the cell fractions; one can only assume that the relation is a genuine one—that under conditions in which the active uptake of chloride by the cell is increased there is also an increase in the rate of transfer of both potassium and chloride from the cytoplasm to the vacuole.

It is not proposed in this paper to go into the details of this link between M_1 and M_2. Consideration of the mean fluxes serves to establish such a link, but throws only limited light on its nature. The relation between M_1 and M_2 in individual cells suggests that the link is a much more intimate one than emerges from the results quoted here, but the detailed interpretation will be discussed in a later paper.

From the present results it is suggested that two separate processes are involved in the active accumulation of salts in the vacuole—an active chloride uptake into the cytoplasm and the subsequent transfer of salt from there to the vacuole. The process of transfer to the vacuole of both potassium and chloride seems to be correlated with the initial uptake of chloride and to be metabolically linked.

A similar distinction between entry of chloride to the cytoplasm and its transfer to the vacuole was also drawn by Arisz (1953), as a result of his work on chloride accumulation in *Vallisneria* leaves, and both processes were said to be metabolically linked. Robertson (1960) suggested that the initial site of ion accumulation might be the mitochondria, and that the ions thus accumulated might be later discharged into the vacuole as the mitochondria came temporarily in contact with the tonoplast. In *Nitella* all three of the processes of active potassium uptake, active chloride uptake, and transfer of ions from cytoplasm to vacuole, seem to be mainly light-driven. It is not possible to imagine an analogous sequence of uptake of ions by chloroplasts and subsequent contact discharge, as the chloroplasts in *Nitella* are stationary and remote from the tonoplast. Sutcliffe (1960, 1962) has suggested that proteins may act as ion carriers, and that membrane synthesis, folding, and vesiculation may be involved in the movement of ion-protein complexes within the cytoplasm and their discharge to the vacuole.

The present work throws no light on the nature of the active uptake of chloride or of the process of discharge of ions to the vacuole, but merely indicates the existence of two such processes. It suggests that it is important to distinguish between uptake of chloride into a phase of the cytoplasm and subsequent transfer to the vacuole, in assessing the effects of metabolic agents

on the over-all process of accumulation. It is not enough to measure net uptake of either an anion or a cation and to equate this to the process of "salt accumulation," as has been done so frequently in the literature. At least in *Nitella* the ionic state of the cell is the result of two independent active transport systems—a cation transport regulating the balance of sodium and potassium, and an anion transport maintaining the high total salt concentration of the cell. But further than that, it now appears that two distinct processes are involved in the ultimate accumulation of salts in the vacuole. In the complications of multicellular plant tissues it is difficult to separate these various processes, but technical difficulties do not any longer justify the assumption that it is enough to measure net accumulation in the tissue as a whole, of either an anion or a cation presented in the external solution. There is no reason to suppose that cells of higher plant tissues will be simpler than those of *Nitella*, and it seems reasonable to regard the two systems as probably similar in general organisation and in basic biochemical properties, until they are proved to be different.

It is hoped that further work will clarify the nature of the process by which salt is transferred from the cytoplasm to the vacuole. The effects noted here would be consistent with some of the views on the origin of vacuoles now current among electron microscopists. It has been suggested (see for example the review by Buvat (1963)), that vacuoles are differentiated from the endoplasmic reticulum, or from a part of it; it is suggested that dilatation of parts of endoplasmic reticulum may give rise to small vesicles, which later merge with the large central vacuole. If such dilatation were linked to the initial active uptake, or if both were similarly dependent on cell metabolism, then a correlation between the initial active uptake and a transfer of salt to the vacuole would indeed be observed.

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