ION TRANSPORT IN NITELLOPSIS OBTUSA

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ABSTRACT

The distribution and rates of exchange of the ions sodium, potassium, and chloride in single internodal cells of the ecorticate characean, *Nitellopsis obtusa*, have been studied.

In tracer experiments three kinetic compartments were found, the outermost "free space" of the cell, a compartment we have called "protoplasmic non-free space", and the cell sap.

The concentrations in the vacuole were 54 mM Na^+, 113 mM K^+, and 206 mM Cl^-. The steady state fluxes across the vacuolar membrane were 0.4 pmole $Na^+/cm.^2$ sec., 0.25 pmole $K^+/cm.^2$ sec., and 0.5 pmole $Cl^-/cm.^2$ sec.

The protoplasmic Na/K ratio is equal to that in the vacuole but protoplasmic chloride is relatively much lower. Osmotic considerations suggest a layer 4 to 6 μ thick with sodium and potassium concentrations close to those in the vacuole. The fluxes between protoplasm and external solution were of the order of 8 pmoles $Na^+/cm.^2$ sec. and 4 pmoles $K^+/cm.^2$ sec.

We suggest that the protoplasm is separated from the cell wall by an outer protoplasmic membrane at which an outward sodium transport maintains the high K/Na ratio of the cell interior, and from the vacuole by the tonoplast at which an inward chloride transport maintains the high vacuolar chloride. The tonoplast appears to be the site of the principal diffusion resistance of the cell, but the outer protoplasmic membrane probably of the main part of the potential.

INTRODUCTION

There are practically no studies on the ion permeability of plant cells and tissues which are comparable—in the details elucidated—with those made by animal physiologists on nerve, muscle, and erythrocytes. The plant cell with its cell wall, large central vacuole, and thin layer of protoplasm is sufficiently complicated to make such a study difficult but in the plant physiologists' favourite material—roots, storage tissue, etc.—these difficulties are added to by morphological complications. It has thus been possible less often in plant systems to express the results of an experiment in fully quantitative terms—as an ion flux in pmoles (10^{-12} moles) per sq. cm. per second across a well defined morphological boundary separating two phases in which the electrochemical activities of the ion are known. This is the general aim of our work.

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Ion uptake by a plant tissue is generally agreed to be a two stage process, a physical non-metabolic entry followed by metabolic transport across a selective barrier, but the nature of these stages and their association with specific morphological features of the tissue cell are very controversial. Some would confine the non-metabolic uptake to the cell walls and extracellular spaces (Levitt, 1957) while others consider that much of the protoplasm is "open" to free diffusion of the ions (Briggs (1957); Briggs and Robertson (1957)). Arisz (1956) suggests that, although the main barrier to ion penetration lies in the tonoplast, entry into the protoplasm is metabolically determined and does involve passage through a diffusion barrier. Electron micrographs (Mercer *et al.* (1955); Farrant *et al.* (1956)) show typical "membranes" at the tonoplast and around the mitochondria but none at the outer boundary of the protoplasm. However, direct observation of the existence of a plasmalemma is hampered by the presence of a cell wall. Transport into the vacuole is generally agreed to be an active process dependent on metabolism (Hoagland and Broyer, 1936) in which the participation of some form of carrier molecule is accepted (Osterhout, 1935). Considerable selectivity towards similar ions has been found, both in early studies and in more recent work (Collander (1936, 1939); Epstein and Hagen (1952); Sutcliffe (1957); MacRobbie and Dainty (1958)). Salt accumulation requires the active movements of either cations or anions but not necessarily of both, for the passive movement of one type of ion can take place down the potential gradient set up by the active transport of the ions of opposite sign. Direct determination of the electrochemical activities of ions on both sides of a permeability barrier across which active transport takes place is needed to settle this question of which ions must be actively transported.

We decided, after an experimental study of ion transport in the marine alga *Rhodymenia palmata*, that quantitative studies on ion movements in plant cells have more chance of success if the complications of the morphology of single plant cells are not supplemented by those due to multicellular organisation. The giant cells of *Nitellopsis obtusa*, a brackish water, ecorticate, characean seemed the most suitable cells available to work with.

Much previous work has been done on members of this group, particularly by Collander, Osterhout, Blinks, Umrath, and others (Collander (1936, 1939); reviews by Osterhout (1955), Blinks (1955), Umrath (1956)). However, these studies have been confined chiefly to chemical measurements of normal concentrations in cell sap and the net uptake from unfamiliar media under a variety of conditions, and electrical measurements of potentials and resistances. Very few kinetic studies have been carried out using tracer techniques. Only these latter will be mentioned here.

Brooks (1940, 1951), whose experiments were few in number and not altogether satisfactory, found that radioactive sodium and potassium entered the protoplasm of *Nitella* more readily than they crossed the tonoplast into the vacuole and that after 6 hours there was no measurable penetration into the

sap. Hoagland and Broyer (1942) measured the rate of penetration of rubidium into *Nitella* and also concluded that the tonoplast was the main barrier to ions. Holm-Jensen, Krogh, and Wartiovaara (1944) measured "permeability" constants, using Na^{24} and K^{42} in *Nitella* and *Nitellopsis*; they concluded that the tonoplast is *more* permeable than the plasmalemma, but a critical analysis of their results indicates the opposite. These kinetic studies are thus fragmentary and are not sufficiently detailed and extensive to give a satisfactory quantitative description of the ion distribution and its kinetics in characean cells. Our work is an attempt to initiate such a study.

Methods

Nitellopsis obtusa was obtained from Snappertuna, Finland, where it occurs in brackish water of chloride concentration 20 to 50 mM. The plants used for the experiments were kept in tanks of aerated brackish water (sea water diluted 15-fold) under cool, not too bright, conditions. They survived such storage for 4 to 6 months before cells died in serious numbers. Rapid protoplasmic streaming and high turgor are sensitive indicators of the health of the cell and only cells showing both these properties were used. Experiments were done on single internodal cells, length 4 to 10 cm. and diameter 500 to 800 μ; such isolated cells appear to be no less able to survive than the complete uprooted plant. The cells were handled by the cut ends of the neighbouring cells or by tying a thread loosely round the end of the cell where the node prevented its slipping off; this prevented damage to the cell which is very easily destroyed by bending, pinching, etc. in direct handling.

Solutions.—An artificial brackish water similar to the normal external medium of the plant was prepared by combining suitable proportions of N/10 or N/20 NaCl, N/20 KCl, distilled water, and a solution which was equivalent to artificial sea water minus its NaCl and KCl content. The final solution contained the ions of diluted artificial sea water (recipe in Hodgkin and Keynes (1955)) in their correct proportions and could be conveniently labelled by the substitution of radioactive NaCl or KCl in its preparation. Its composition was (mM): Na, 30; K, 0.65; Ca, 0.67; Mg, 3.40; Cl, 35; SO_4, 1.80; phosphate, 0.09. Cells were isolated from their neighbours and stored in a shallow dish for at least a week in this solution under laboratory conditions of light and temperature before use in experiments. Potassium-enriched solutions were prepared by substituting for part of the NaCl an equivalent amount of KCl, thus keeping Na + K constant.

Na^{24} and K^{42} were each obtained from Atomic Energy Research Establishment, Harwell, as the spectroscopically pure carbonates and were titrated with N/20 HCl to pH 7.0. An artificial brackish water was then prepared in the usual way but with the substitution of N/20 $K^{42}Cl$ or N/20 $Na^{24}Cl$ for an equivalent amount of the inactive salt. The activity of these solutions was high, particularly that of Na^{24}, being as much as 150 μc./ml. at the beginning of an experiment. During long experiments with solutions labelled with radioactive sodium the total dose was about 1000 rads. It should therefore be kept in mind that the permeability might not be normal under these conditions, though cells were capable of surviving for several months after larger doses than this, with their protoplasmic streaming apparently unaffected.

Na^{22} was also used in some experiments in which the activity of the solution was

much less (about 10 μc./ml.) but as the experimental time of uptake was correspondingly longer the total dose was no less in the Na^{22} experiments than in the Na^{24} experiments. The Na^{22}-labelled solution was prepared by adding $Na^{22}Cl$ to 10 ml. inactive artificial brackish water, thereby increasing the sodium concentration by not more than 3 per cent.

Br^{82}-labelled solutions were prepared by the substitution of $NaBr^{82}$ for a part of the NaCl in the uptake solution; pure, radioactive, NH_4Br, obtained from A.E.R.E., was converted to NaBr by the addition of a slight excess of NaOH, boiling to dryness to remove the ammonia, and back-titration of the excess alkali with HCl to pH 7.0. (The bromide content of this solution was taken equal to the total halide although, strictly, chloride is also present to the amount of the excess NaOH originally added.)

Cl^{36} was obtained from Amersham as 1.65 N HCl; this was titrated to pH 7.0 with N NaOH and the solution diluted to a concentration of 50 mM. The active solution was then prepared from the usual constituents but N/20 $NaCl^{36}$ was used in place of inactive NaCl.

Isotope Experiments.—The procedure was very similar to that used in the experiments on *Rhodymenia* (MacRobbie and Dainty (1958)), except that the same type of washing-out tube was not used. The cells were soaked in radioactive solutions to label the internal ion content; in experiments with short lived isotopes the time of uptake for cells on which a washing-out experiment was done was not usually more than 15 hours, but when only the influx was measured, uptake times of up to 90 hours were possible. With the longer lived isotopes Na^{22} and Cl^{36}, when the decay of radioactivity was no longer the limiting factor, cells were soaked for up to 1 to 2 months in labelled solutions before the start of the washing-out. For the shorter uptakes the cells were under continuous illumination (laboratory fluorescent lighting) and at room temperature, but in long experiments the conditions during the prolonged soaking were less well defined; lighting followed the normal day-night pattern. In general temperature was not controlled except in the Cl^{36} experiment when it was maintained at 20 $\pm$ 1°C. Usually the uptake was used only to label the cells; when it was used to calculate an influx the illumination was kept constant.

During washing-out experiments the cells were continuously illuminated and at a room temperature of 17–20°C. In these experiments the cells were in flat, covered perspex dishes in a 0.5 cm. deep layer (10 ml.) of inactive brackish water and the cell was transferred to fresh solution by lifting it from one dish to another either by the cut end of the neighbouring cell or by a loose thread. After the first few minutes of washing-out the frequency of solution changes was gradually decreased from 10 minutes to 2 hours over the first 6 hours of the experiment; later during the slow exchange from the cell sap the time intervals were 2 to 10 hours. A calculation of the rate of removal of labelled ions by diffusion in the bathing solution showed that the ratio of labelled to unlabelled ions in the immediate vicinity of the cell did not rise above 2 per cent during either phase of the exchange. It was therefore considered that the rate of loss of radioactivity, except during the first rapid exchange from the free space of the cell, was slow enough for rapid stirring to be unnecessary.

At the end of the washing-out experiments the cell was measured, the diameter microscopically to about 1 per cent and the length to about 2 per cent from a tracing of the cell on a sheet of paper. The cell was then ashed in 10 ml. of cold N HNO_3,

or in some experiments a sap sample was removed for counting and chemical estimation and the rest of the cell ashed. (It was checked that this simpler procedure gave the same results as complete ashing in hot concentrated H_2SO_4 and concentrated HNO_3.)

Liquid counting was used in all but one experiment as described in the previous paper (MacRobbie and Dainty (1958)). The specific activity of the Cl^{36} was rather low for satisfactory liquid counting and in one experiment the chloride samples were precipitated as AgCl on a disc of filter paper and counted by an EHM 2 end-window counter, with an efficiency 5 times higher than M6 liquid counting. Standards were counted under the same conditions for comparison.

Sap Samples.—Sap samples were obtained by rinsing and lightly blotting the cell, cutting off the end with sharp scissors, and gently squeezing out the sap on to a teflon square. The drop of sap was then drawn up into a micropipette and was ready for analysis. Its volume was determined either by using a graduated micropipette and a travelling microscope, or by using a pipette of drawn-down glass with one mark, from which an equal volume of a known standard solution was delivered for comparison. In general 5 to 10 μl. of colourless sap was obtained by this method. This volume of sap was ample for the determination of Na and K by flame photometry and for determination of radioactivity.

Chemical Analysis.—The Na and K in the sap samples were determined using an EEL flame photometer. The 5 to 10 μl. sap sample was added to 5 ml. of distilled water and this solution was analysed by comparison with a standard prepared in a similar way from an artificial sap solution, whose Na and K concentrations were chosen to be similar to those in sap. Various checks indicated that the results were reliable to better than 2 per cent. The Na and K in whole cell were also determined in a number of cases either by "wet-ashing" in N HNO_3 or by dry-ashing in a platinum crucible at 450°C. for 24 hours, followed by flame photometry on suitable solutions.

Chloride was determined to 1 to 2 per cent by electrometric titration by a method similar to that described by Ramsay, Brown, and Croghan (1955).

RESULTS

Chemical Analysis of Sap

Throughout the course of the experiments a large number of sap analyses—of Na, K, and Cl—were done and the results are summarised in Table I, in which the external concentrations are also given for comparison. (Results are quoted in the form: mean ± standard error of mean (number of determinations).)

Isotope Experiments

The results of the washing-out experiments were treated in the standard manner; the logarithm of the amount of radioactivity left in the cell, or the logarithm of the rate of loss of radioactivity, was plotted against time, a plot in which uniform exchange from a single compartment gives a straight line. In the experiments with *Nitellopsis* the washing-out could be split into three phases and three compartments with distinct efflux rates could be distinguished.

At long times the washing-out curves were linear if plotted in this way and the bulk of the ions of the cell was included in this fraction, but for several hours at the start of the experiment the rate of loss of activity was considerably greater than that of the ions in this slow fraction. Subtraction of the slow fraction from the total activity in the cell gave a second straight line on a semilogarithmic plot, suggesting the presence of a second compartment in the cell with a faster rate of exchange. In addition there was a very rapid loss of radioactivity in the first minute or so which is ascribed to rapid exchange with the free space ions. (The assignment of this very fast fraction to the free space is based on the very high rate of exchange, the fact that the rate of exchange is independent of temperature, and the ratios of the amounts of Na, K, and Cl in this fraction. However, because of certain technical difficulties, we have not yet been able to obtain the absolute amounts of the ions in this free space fraction and hence cannot say what volume of the cell it occupies. Work is being continued on this problem and we hope to publish the results in a later paper.) This present paper

TABLE I

Concentrations of Na, K, and Cl in the Sap and in the External Solution

	Na	K	Cl
Sap concentration range, *mM*.	26.3 – 77.0	74 – 159	143 – 249
Mean sap concentration, *mM*.	53.9 ± 1.6 (63)	113 ± 2 (69)	206 ± 3 (45)
External solution, *mM*.....	30	0.65	35

will be concerned with the two "slower" compartments of the cell, which will be discussed separately.

Slow Compartment: Cell Sap

The slow fraction may be fairly definitely associated with the ions of the cell sap; this will be obvious from the results as they are presented but the reasons on which this conclusion is based may be summarised here. These are that the amounts of Na, K, and Cl in the slow fraction of a washing-out experiment correspond with those estimated chemically in the sap, and that the influx into the slow compartment does not differ significantly from the influx into the cell sap determined by direct counting of samples of radioactive sap isolated from a labelled cell.

Potassium Influx.—This was determined, both from the activity in the slow fraction and from the activity of sap isolated from the cell, in the normal external medium (0.65 mM K) and also in external solutions of higher potassium concentration. (Solutions of potassium concentration up to 4 mM were prepared in which the sodium concentration was lower than normal by the amount of the increase in potassium concentration.) The results obtained are summarised in Table II.

Potassium Efflux.—Measurements of the efflux of potassium from this compartment were hampered by the slowness of the ion exchange and the short half-life of the isotope K^{42} (12.4 hours). For this reason the cells on which the efflux was measured were soaked in a radioactive solution of higher potassium concentration than normal so as to produce a reasonably high specific activity of potassium at the beginning of a washing-out experiment.

TABLE II

Potassium Influxes, pmoles/cm.² sec. into the Sap from Solutions of Various K Concentrations

K concentrations	0.65 mM	1.3 mM	2.1 mM	4.2 mM
Range, *pmoles/cm.² sec.*	0.12 – 0.41	0.7 – 1.95	2.8 – 4.6	4.8 – 8.5
Influx Mean, *pmoles/cm.² sec.*	0.22 ± 0.02(15)	1.5 ± 0.2(7)	3.8 ± 0.2(9)	6.5 ± 0.4(10)

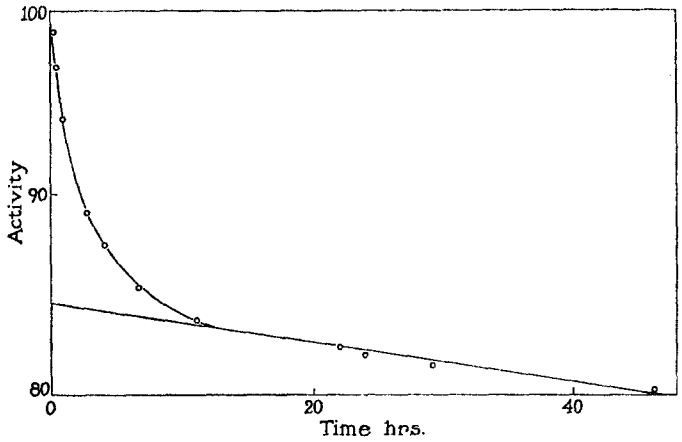

FIG. 1. Exchange of potassium in the cell sap.

Cells were labelled in K^{42} solutions of potassium concentrations 2.1 mM or 4.2 mM and were washed out into normal brackish water of potassium concentration 0.65 mM. One of the washing-out curves is shown in Fig. 1. The mean values of k, the efflux rate constant, for the slow fraction are given in Table III; since k decreased during this time to a steady value, this lower, steady, value is also given. The values of kV/A (V is the cell volume and A the surface area) are also given but, since total K was not determined in each case, the individual effluxes (kV/A times C_i, the internal potassium concentration) cannot be determined.

The mean value of kV/A (calculated from the final, steady, value of k) is 0.89 ± 0.07 (6) $\times 10^{-5}$ cm./hr. which, combined with the mean value of 113 ± 2 mM for the K concentration of the sap, gives an efflux of 0.28 ± 0.03 pmole/cm.^{2}sec. If the mean efflux over 0 to 50 hours is calculated, its value is 0.59 pmole/cm.^{2}sec. Thus the efflux during 50 hours in 0.65 mM K solution after an uptake of about 16 hours in a high K solution is just over twice the normal influx, but by the end of this period the efflux has decreased until it is not significantly different from the normal influx. It seems therefore that, under normal conditions, the fluxes into and out of the sap are equal to each other (and are about 0.25 pmoles K/cm.^{2}sec.) and thus there is flux equilibrium.

Sodium Influx.—The influx of sodium into the sap was determined by means of Na^{24} in the same way as has been described for the potassium influx. Solutions of the same chemical composition as those for which the potassium influx

TABLE III

Exchange of K between Sap and Various External Solutions

K concentration in uptake solution	Influx in uptake solution	K concentration in washing-out solution	Mean k 0–50 hr.	Final k	kV/A
mM	pmoles/cm.^{2} sec.	mM	10^5 hr.$^{-1}$	10^5 hr.$^{-1}$	10^5 cm./hr.
4.2	5.2	0.65	1.1	0.4	0.63
4.2	6.9	0.65	1.1	0.6	1.01
4.2	4.8	0.65	1.1	0.5	0.83
2.1	4.3	0.65	1.1	0.6	0.94
2.1	3.5	0.65	1.1	0.8	1.11
2.1	2.8	0.65	1.1	0.45	0.80

was determined were used. Table IV summarises the values obtained for external solutions of K concentration 0.65 mM, 2.1 mM, and 4.2 mM and Na concentrations 30 mM, 28.55 mM, and 26.45 mM. From these figures an increase in potassium concentration does not appear to have any very marked effect on the sodium influx; the influxes from 0.65 mM and 2.1 mM K solutions are not significantly different and the number of determinations at 4.2 mM is too few for any conclusions to be drawn.

Sodium Efflux.—The efflux of sodium from the sap has been investigated by means of both Na^{24} and Na^{22}. Experiments with Na^{24} are limited by the short half-life of the isotope (15 hours) but on the other hand avoid the difficulties of flame photometry of radioactive samples containing the long lived Na^{22} (half-life 2.6 years). In the Na^{24} experiments, cells were soaked in labelled solution for 13 to 16 hours and the rate of loss of activity from groups of 4 to 5 cells was then followed for 80 hours. Even for 4 to 5 cells the counting rates were very low and some of the counting errors were as high as 10 per cent. After the washing-out the cell was dry-ashed and the total Na determined by flame photom-

etry. The sap Na was determined by subtraction, from the total Na, of the fast Na; the amount of the latter could be determined from the washing-out curve since it can be assumed to have completely exchanged during the uptake time of 13 to 16 hours. The influxes and effluxes determined from these experiments are necessarily approximate because they are averages for groups of cells; the averaged results of five experiments which gave five influx values and three efflux values were: mean Na influx, 0.39 ± 0.04 (5) pmole/cm.²sec., mean Na efflux, 0.60 ± 0.02 (3) pmole/cm.²sec.

The efflux rate constants were also determined for a number of cells using Na^{22} but, as no measurements of total sodium were made, this did not give values of the efflux in the individual cells. An approximate value only of the efflux can be found from the rate constant and the mean Na concentration in the sap. As the cells were soaked in Na^{22} solution for some weeks under rather variable conditions of light and temperature, no influx values could be calculated from the amount of uptake during this time. The efflux rate constants were

TABLE IV

Sodium Influxes into the Sap from Solutions of Various K Concentrations

K concentrations	0.65 mM	2.1 mM	4.2 mM
Range, *pmoles/cm.² sec.*	0.09 – 0.50	0.10 – 0.53	0.50 – 0.52
Influx			
Mean, *pmoles/cm.² sec.*	0.27 ± 0.03 (20)	0.32 ± 0.06 (7)	0.51 (2)

determined over the period 10 to 120 hours of the washing-out process. Three experiments gave values for $10^5 kV/A$ of 1.7, 2.5, 2.6 (cm./hr.) and combining these with the mean concentration of sodium in the sap (54 mM) gives an average efflux value of about 0.35 pmole/cm.²sec., which, in view of the uncertainties in obtaining this figure, cannot be considered as disagreeing with the Na^{24} estimate.

Chloride Influx.—Cells were soaked for 40 to 100 hours in Cl^{36}-labelled solution and the influx was determined either by direct counting of sap samples or from the amount left in the cell after washing in inactive solution for 24 hours to remove the activity in the fast fractions. The results of nine experiments (four by sap isolation and five from the slow component) gave Cl influxes ranging from 0.27 to 0.73 with a mean value of 0.45 ± 0.04 (9) pmole/cm.²sec.

Chloride Efflux.—The very low specific activity of the Cl^{36} available made determination of the efflux of chloride from the sap difficult. In the first experiments the cells were soaked in the Cl^{36}-labelled solution for 6 weeks. Such prolonged soaking was necessary in order to introduce sufficient activity into the cell sap but unfortunately, for a reason which remains unknown, the mortality rate in this solution was high. At the end of the 6 week period some twenty cells

survived and washing-out experiments were done in groups of 4 to 5 cells. All these cells showed protoplasmic streaming and were apparently healthy but the possibility remains that they were not entirely normal. The average values of $10^5 kV/A$ for four groups of cells were 1.1, 0.9, 2.6, 1.6 (cm./hr.) and if these are combined with the average Cl concentration in the sap (206 mM) effluxes of 0.63, 0.51, 1.5, 0.92 (pmole/cm.^{2}sec.) are obtained. Rough influx values were also calculated for the last three groups of cells; they were 0.24, 0.4, 0.4 (pmole/cm.^{2}sec). These figures suggest that there is a net loss of chloride from the cell sap under these conditions although it is small enough to produce only very slow changes in the internal chloride concentration. An approximate calculation shows that, at the end of a 6 week period, the cell chloride had probably decreased by 20 to 30 per cent and therefore the above efflux figures are likely to be an overestimate.

Another set of experiments was done with a Cl^{36} solution prepared in exactly

TABLE V

Mean Value of λ_0 for Bromide and Chloride

Halide ..... K concentration.....	Bromide 0.65 mM K	Bromide 2.1 mM K	Bromide 4.2 mM K	Chloride 0.65 mM K
Mean $10^5 \lambda_0$, *cm./hr.*.....	1.6 ± 0.2(10)	3.8 ± 0.6(3)	10.7 ± 0.7(8)	4.6 ± 0.4(9)

the same way and in which the cells were soaked for 1500 hours and washing-out experiments were done on 1 to 2 cells. This uptake solution was quite harmless and the cells survived in it quite as well as in the unlabelled medium. Greater counting accuracy was achieved by solid, end-window, counting of $AgCl^{36}$. Five experiments gave values of $10^5 kV/A$ ranging from 0.81 to 0.98 (cm./hr.) and, combining these results with the mean chloride concentration in the sap (206 mM), led to a mean Cl efflux of 0.52 ± 0.01 (5) pmole/cm.^{2}sec. No influx determinations were made on these cells.

Bromide Influx.—Since the specific activity of Cl^{36} was so low the movements of halide into the cell were also investigated by means of Br^{82}. (Time was allowed for the short lived Br^{80} (18 minutes and 4.4 hours) to decay before any counting was attempted.)

The influx of bromide into the sap was rather variable and the reason for the wide scatter of the results is not known. Since not all the chloride in the normal solution was replaced by active bromide and the external bromide concentration varied in different experiments, the most significant quantity obtained from these influx measurements is the "permeability" constant, λ_0 (cm./hr.), equal to the influx divided by the external bromide concentration. This allows the behaviour of bromide to be compared with that of chloride. Some of these experiments were done with solutions of different K concentration; the total

halide concentration was 35 mM in each case. The results are summarised in Table V in which λ_0 for Cl is given for comparison. From these results it appears that bromide is not a satisfactory tracer for chloride and that it enters the sap less readily than chloride.

Rubidium Fluxes.—The rates of entry and loss of rubidium were determined by means of Rb^{86}. Cells were soaked for 1150 hours in an artificial brackish water containing 1 mM $Rb^{86}Cl$ in addition to the normal constituents. Washing-out experiments were then done in the usual way. The experimental results are given in Table VI, together with some comparative figures for potassium. The influx of rubidium is very low compared with that of potassium and the rate of washing-out of Rb is lower than that of K although the difference is less than in the case of the influx.

TABLE VI

Movements of Rb to and from the Cell Sap, Together with Comparable Figures For K

	Influx	$10^5\lambda_0$	10^3k	$10^5kV/A$
	pmoles/cm.² sec.	*cm./hr.*	*hr.⁻¹*	*cm./hr.*
Rb 1 mM	0.031	11.2	0.25	0.45
	0.028	10.1	0.17	0.25
	0.049	17.6	0.42	0.66
K, 0.65 mM	0.22	122	0.5	0.89
K, 1.3 mM	1.5	415	2.0	3.6

λ_0, the "permeability" constant in cm./hr. for inward movement, equal to the influx divided by the external concentration. k, the rate constant for washing-out, obtained from the slope of the semilogarithmic plot of the activity in the cell against time. kV/A: the permeability constant in cm./hr. for outward movement.

Protoplasmic "Non-Free" Space

As pointed out earlier a *Nitellopsis* washing-out curve is the sum of two exponentials and a diffusion curve or, approximately, the sum of three straight lines when the curve is plotted logarithmically. After subtracting the "slowest" exponential we obtain, in general, a curve similar to that of Fig. 2, which is clearly the sum of an exponential and a very steep curve. We consider that the slowest exponential corresponds to efflux from the cell sap, as indicated above, and that the ions represented by the remainder of the curve are outside the cell sap. Those in the very steep part we identify with the ions of the "free space." We consider the ions in the middle fraction—half-time of the order of an hour—to be in a compartment we shall call "protoplasmic non-free space." One difficulty in discussing this compartment is that its volume is not known. For presentation of the results we shall use the amount of ion divided by the cell volume but this is an arbitrary procedural choice and we shall discuss later what the volume of this compartment is likely to be.

From washing-out experiments with Na^{24} and Na^{22}, the amount of Na, expressed as the amount per liter cell volume, and the half-time for exchange found from the slope of the semilogarithmic plot of this fraction, were found to be 0.8 ± 0.1 (18) mM and 24 ± 2 (18) minutes. The results, expressed in this way, were very variable; the Na concentrations ranged from 0.25 to 2.2 mM and the half-times from 11 to 67 minutes.

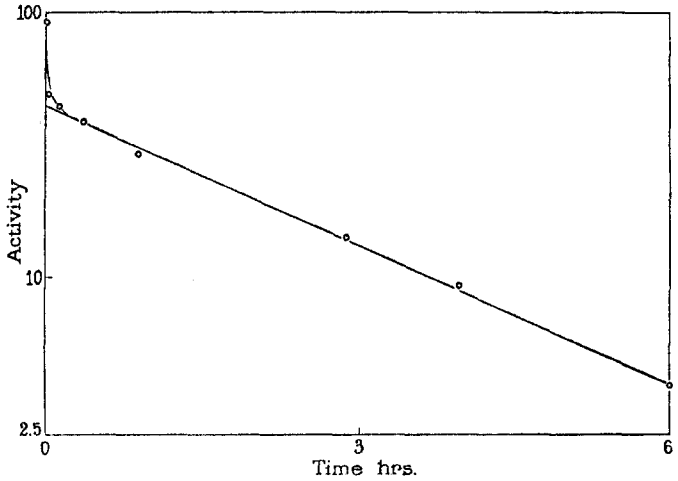

FIG. 2. Exchange of potassium in protoplasmic non-free space.

TABLE VII

Potassium in Protoplasmic Non-Free Space

Uptake solution	Amount of K/liter cell volume	$T\frac{1}{2}$ at 20°C. (hrs.) washing-out into 0.65 mM K	$T\frac{1}{2}$ at 2°C. (hrs.) washing-out into 0.65 mM K
(*mM K*)	*mM*		
0.65	1.4 ± 0.2 (8)	1.65 ± 0.25 (3)	11.35 ± 0.05 (3)
2.1	4.6 ± 0.2 (3)	1.64 (2)	
4.2	5.7 ± 0.7 (7)	1.89 ± 0.10 (3)	

Similar experiments were done with K^{42}. However, in this case the uptake solutions, for labelling the cells, were of various K concentrations; washing-out was done into the normal 0.65 mM K solution. The results are summarised in Table VII.

The relative amounts of Na and K in this compartment are of considerable interest. The above experiments indicate a ratio of K/Na of 1.8, but this ratio is more readily determined by an experiment in which Na^{22} and K^{42} are used simultaneously, when the wide variations among the cells do not obscure the results. Cells were soaked in a solution labelled with Na^{22}, K^{42}, and Br^{82} for 12 to

15 hours and then washing-out experiments were carried out from which the amounts of all three ions in this fraction of the individual cell were determined. The amounts of Na and K and their ratio for three cells are given in Table VIII. It is clear that there is about twice as much potassium as sodium in the protoplasmic non-free space. (The external K/Na ratio is 0.022.)

The amount of rubidium taken up in this compartment from an external solution containing 1 mM Rb^{86} was also determined from the washing-out curves in inactive 1 mM Rb solution after an uptake of 1150 hours. Four experiments gave mean values of 0.21 ± 0.07 (4) mM for the amount of Rb per liter cell volume, and 1.37 ± 0.22 (4) hour for the half-time.

Very few estimates of the amount of chloride or bromide in this fraction have been made. Five determinations of the amount of chloride were made from the activity, in excess of the loss from the sap, lost between 2 minutes and 7.5 hours of a washing-out experiment. The mean result was that the amount of chloride per liter cell volume was 0.32 ± 0.07 (5) mM. The specific activity was too low

TABLE VIII

Amounts of Na and K and Their Ratio in Protoplasmic Non-Free Space, Determined Simultaneously on Same Cell

Amount of Na/liter cell volume	Amount of K/liter cell volume	K/Na
mM	*mM*	
0.68	1.8	2.6
0.60	0.92	1.5
0.25	0.47	1.9

for convenient determination of the rate of exchange of this fraction by means of Cl^{36}. A number of experiments with Br^{82} indicated that the half-time for exchange of "halide" was between 20 and 40 minutes.

DISCUSSION

The most straightforward interpretation of the main results of our experiments is that external ions are first exchanged with free space ions which are distributed according to a Donnan equilibrium; then exchange takes place between free space ions and protoplasmic non-free space ions and finally the protoplasmic non-free space ions exchange with ions in the vacuole across the tonoplast. The latter two exchange processes are not necessarily "passive," physical, processes; our results on fluxes, amounts, and concentrations enable us to decide which ions are probably passively exchanged and which involve some "active" transport.

The figures for the ion concentrations in the sap, together with the observation that the tonoplast is permeable to ions in some form, imply the existence in the cell of a mechanism of active transport for some of the ions, although the

sites of this transport cannot be determined from this information alone. If an ion is moving *passively* from an external solution in which its concentration is C_o to an internal solution in which its concentration is C_i, then the net flux (inwards) of the ion is given by:

$$M = P\ \{C_o - C_i \exp\ (zFE/RT)\} \tag{1}$$

in which M is the flux in moles/cm.^{2}sec.; z is the charge, in units of the electron charge, on the ion; F is the faraday; R the gas constant, and T the absolute temperature; E is the electrical potential difference between the inside and outside solutions; P is a permeability factor depending on the properties of the membrane separating the two solutions (see chapter 11 in Johnson, Eyring, and Polissar (1954)). Equation (1) is the equivalent, for ions, of Fick's diffusion equation. We are making the usual approximation of replacing activities by concentrations; this should lead to no serious error when dealing with two aqueous solutions such as the external, brackish solution and the sap.

TABLE IX

Ion Concentration Potentials (E) in the Sap

Ion	C_o/C_i	$E = \frac{58}{z} \log \frac{C_o}{C_i}$ mv.
Na^+	0.556	−15
K^+	0.00575	−130
Cl^-	0.170	+45

Our results indicate, as would be expected, that when a *Nitellopsis* cell is bathed by its normal external medium the influx of any ion into the sap equals the efflux; *i.e.*, the net flux is zero. Thus any ion moving passively between the external solution and the sap must have its concentrations governed by the equation:

$$M = P\ \{C_o - C_i \exp\ (zFE/RT)\} = 0$$
$$i.e.\ E = \frac{RT}{zF} \ln \frac{C_o}{C_i} = \frac{58}{z} \log \frac{C_o}{C_i} \text{ mv.} \tag{2}$$

In Table IX we give the values E (the ion concentration potential) should have if the ions concerned move passively under the action of the purely physical forces of chemical and electrical potential gradients. Since the vacuolar potential must have a unique value, it is clear that at least two of the three ions must be actively transported between external solution and sap.

Recently in this department Dr. Williams and Dr. Johnston measured the potential difference between the vacuole of *Nitellopsis* and the normal brackish external medium. Their results range from 120 to 200 mv. with the vacuole negative. It would thus appear that whereas it is possible that the potassium ion in the vacuole is in electrochemical equilibrium with the external potassium,

both sodium and chloride are very far from their equilibrium distribution. Thus sodium and chloride must be undergoing active transport and it is clear from an examination of Table IX that sodium is actively transported outwards and chloride actively transported inwards.

The probable sites of these transports in the system of compartments found in the tracer experiments may be deduced from the relative amounts of the three ions in each compartment and the probable relations of the tracer compartments to cell morphology may then be considered. The reasons for the association of the slow compartment with the cell sap have already been given. The volume of the second compartment, the protoplasmic non-free space, is uncertain but some of its properties can be discussed without specifying its volume. The K/Na ratio in this compartment is about 2 and does not differ significantly from the K/Na ratio in the vacuole. This suggests that the sodium-potassium selectivity is a property of the protoplasmic non-free space. We suggest that this selectivity is a property of a membrane separating this space from the free space and that it is due to an outwardly directed sodium pump of the kind postulated by Hodgkin (1951) to explain the same kind of selectivity in nerve and muscle. The ratio (Na + K)/Cl is about 7 and may indeed be much higher as our figure for protoplasmic chloride is likely to include contamination with free space chloride; this figure differs markedly from the vacuolar ratio of 0.8. This, together with our later estimates of ion concentrations in the non-free space, suggests that the inwardly directed chloride pump is located at the tonoplast, between the protoplasmic non-free space and the vacuole.

We may speculate about the size of the protoplasmic compartment from osmotic considerations, since it is presumably in osmotic equilibrium with the vacuole. The only "facts" available are the total amount of monovalent cation—2.2 m. mole/liter total cell volume—and the osmolarity—approximately 400 mM. Reasonable guesses have to be made about whether the balancing anions are fixed or mobile and about the activity coefficients of the monovalent and divalent cations. Depending on these assumptions the values for (Na + K) concentration would range from 120 to 200 mM if the ion activity coefficient were unity and would be correspondingly greater for lower activity coefficients. These figures lead to estimates of protoplasmic non-free space volume ranging from 1½ to 1 per cent of the cell volume (or less if the ion activity coefficients are low). For a typical cell of length 5 cm. and diameter 600 μ the protoplasmic non-free space, if it were spread in a single layer near the cell wall, would be 2 to 3 μ thick (or less if there was appreciable cation binding). This figure for the thickness must be increased if allowance is made for the osmotically inactive solids in the protoplasm; an increase of up to a factor 2 is possible, leading to an estimated thickness of 4 to 6 μ.

Of the several possibilities for the location of the protoplasmic non-free space the most likely seem to be: (*a*) the whole of the protoplasm; (*b*) a 4 to 6 μ

thick layer bordering the tonoplast, the flowing protoplasm perhaps; (*c*) some or all the chloroplasts, mitochondria, etc., which would then be embedded in the so called protoplasmic free space of the cell.

All these possibilities have their virtues: (*a*) would be in conformity with the current picture of external membrane-limited animal cells, although the estimated protoplasm thickness is only just within the range of previous estimates in Characeae, from 5 to 15 μ (Collander (1930), Holm-Jensen, Krogh, and Wartiovaara (1944), Peebles (1956)); (*b*) is compatible with estimates of the thickness of the flowing protoplasm and might fit in with Arisz's symplasm theory; (*c*) has been discussed by Robertson (1956), who gives reasons for considering the mitochondria as the sites of ion selectivity and accumulation. We prefer possibility (*a*) (or (*b*)), partly to conform with the accepted picture of animal cells; also if the protoplasmic non-free space is confined to the mitochondria a substantial proportion of the cations would have to be bound (this is in agreement with Robertson's ideas); in addition our protoplasmic non-free space exchange rates are 50 to 100 times slower than isolated beet mitochondria exchange rates. Finally it is difficult to reconcile the amounts and relative proportions of the various ions in the two phases with the concept of mitochondria as ion carriers across the tonoplast. On the basis of (*a*), a provisional scheme for the normal state is given in Fig. 3 showing the ion concentrations and fluxes in the two compartments. The somewhat speculative concentrations in the protoplasm and fluxes into the protoplasm are given in parentheses. The tonoplast fluxes and vacuolar concentrations are not so uncertain. The scheme assumes the ion pump hypothesis.

On this picture the protoplasm is separated from the cell wall, a Donnan system, the "free space", by an outer protoplasmic membrane which seems to be similar to a typical animal cell membrane, and from the vacuole by the tonoplast, a typical plant cell membrane. At the outer membrane an outward sodium pump, perhaps coupled to an inward potassium transport as has been suggested in animal cells (Hodgkin and Keynes, 1955), maintains the high K/Na ratio of the protoplasm. The fluxes (influx and efflux) of Na and K across this membrane, based on this scheme, are about 8 pmoles Na/cm.^{2}sec. and 4 pmoles K/cm.^{2}sec. Assuming that the Na and K *influxes* are both passive we can deduce a K/Na permeability ratio of about 23. These fluxes are rather lower than those in squid nerve but similar to those in muscle (Hodgkin, 1951). The permeability ratio is also similar to those found in animal cells (Conway (1957); Hodgkin (1951)), and the low protoplasmic chloride implies the existence of indiffusible anions in the protoplasm as in animal cells.

Up to the tonoplast the system seems very similar to the typical animal cell, but the tonoplast is a membrane with very different properties. There is little discrimination between sodium and potassium at this membrane and it is therefore likely that the cation fluxes across the tonoplast are entirely passive. From the fluxes and concentrations, the tonoplast is slightly more permeable to

sodium than to potassium, in marked contrast to the plasmalemma and animal cell membranes which are much more permeable to potassium than to sodium. Also the tonoplast is much tighter to ions than the outer protoplasmic membrane and than animal cell membranes, for the fluxes across the tonoplast are 20 to 100 times lower. The tonoplast must therefore be the site of the principal diffusion resistance of the plant cell. The tonoplast fluxes lead to a calculated value of the electrical resistance between vacuole and external medium of

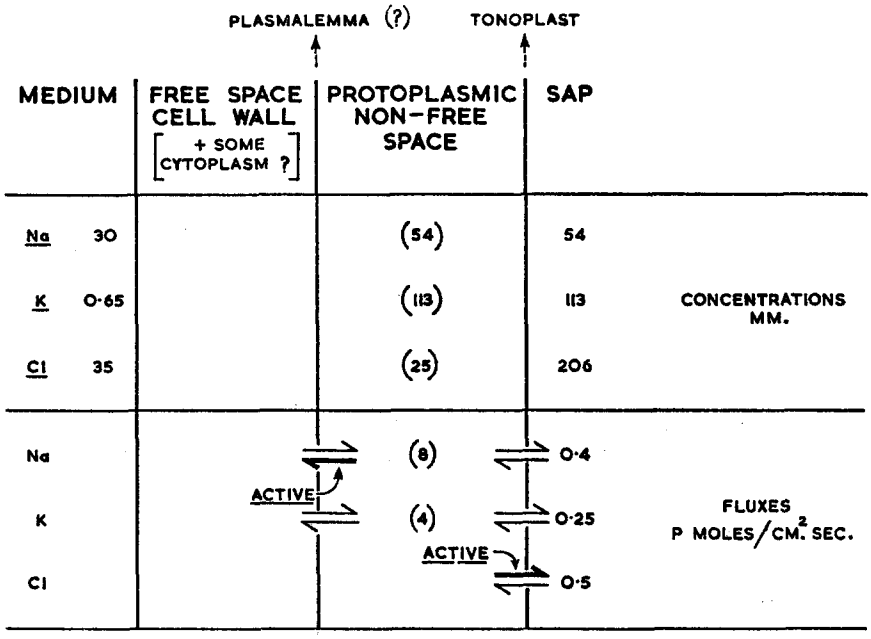

FIG. 3. Provisional scheme for the normal state of the *Nitellopsis* cell showing ion concentrations and fluxes in the two compartments. Protoplasmic figures, shown in parentheses, are uncertain as the volume of this compartment is not accurately known.

about 250,000 ohm cm.^{2}, in agreement with the estimate of Blinks (1930) but not with those of Bennet and Rideal (1954) and Walker (1957).

Some predictions of the partition of the total potential of the cell between the two membranes may be made for the proposed system but so far we have not been able to measure the potential across either membrane separately. Since Na and K appear to be passively distributed across the tonoplast in approximately equal concentrations, there should be only a small potential difference (see equation (2)), and thus the main potential drop would be expected across the external protoplasmic membrane. Walker's (1955) measurements on *Nitella* support this deduction and, if so, it is interesting that the main potential drop and the main resistance seem to be associated with different membranes.

The energy requirements for the proposed system of pumps may be calculated (see Keynes and Maisel (1954)). The minimum work associated with an active sodium efflux of 8 pmoles/cm.^{2}sec. at the outer membrane is about 80×10^{-3} cal./gm. protoplasm per hour and with an active chloride influx at the tonoplast of 0.5 pmole/cm.^{2}sec. is about 9×10^{-3} cal./gm. protoplasm per hour. A total energy requirement of 9×10^{-2} cal./gm. protoplasm per hour should be well within the metabolic capacities of the cell.

The effects of changing the external potassium concentration are very striking. A detailed analysis of the results, which will be published later after further experiments, indicates that they can all be best explained by a general increase of the tonoplast permeability to *all* ions.

The difference in behaviour between potassium and rubidium is rather surprising since the ions are much the same size and are usually considered as biological near equivalents. The results show that the difference is due to the much lower permeability of rubidium, as compared with potassium, at the outer protoplasmic membrane. At the tonoplast it seems to have the same permeability as sodium and potassium. This might suggest that a considerable part of the inward potassium movement at the outer membrane was active and that a linked sodium-potassium pump of the type proposed for squid nerve was operating here.

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