Ion Transport in *Hydrodictyon africanum*

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ABSTRACT The concentrations of K, Na, and Cl in the cytoplasm and vacuole, the tracer fluxes of these ions into and out of the cenocyte, and the electrical potential difference between bathing solution and vacuole and cytoplasm, have been measured in *Hydrodictyon africanum*. If the ions were acted on solely by passive electrochemical forces, a net efflux of K and Cl and a net influx of Na would be expected. Tracer fluxes indicate a net influx of K and Cl and efflux of Na in the light; these net fluxes are consequently active, with an obligate link to metabolism. The effects of darkness and low temperature indicate that most of the tracer K and Cl influx and Na efflux are linked to metabolism, while the corresponding tracer fluxes in the direction of the free energy gradient are not. Ouabain specifically inhibits the metabolically linked portions of tracer K influx and Na efflux. Alterations in the external K concentration have similar effects on metabolically mediated K influx and Na efflux. It would appear that K influx and Na efflux are linked, at least in the light.

INTRODUCTION

Much of the recent work on the ionic relations of algae has been directed towards discovering which ions are actively transported into or out of the cells. By active is meant transport against an electrochemical potential gradient. If flux equilibrium can be shown, the measured potential difference (PD) between the vacuole and the external medium may be compared with the equilibrium potential calculated from the internal and external concentration of a given ion, using the Nernst equation (Briggs, Hope, and Robertson, 1961; Dainty, 1962). Discrepancies between the observed potential and the equilibrium potential calculated from the Nernst equation for any given ion may be attributed to active transport. If isotopic measurements of influx and efflux indicate that the cells are not in flux equilibrium for a given ion, then a more complicated expression must be used, involving the flux ratio expected from the observed electrochemical gradient.

Measurements necessary to specify active transport between two phases are the ion concentrations in the two phases, the PD between the two phases, and the ion flux between the two phases. Work in which these measurements have been made on algal cells includes that of MacRobbie and Dainty (1958

a,b) on *Rhodymenia palmata* and *Nitellopsis obtusa*; Gaffey and Mullins (1958) on *Chara globularis*; Blount and Levedahl (1960) on *Halicystis ovalis*; Hope and Walker (1960, 1961) and Hope (1963) on *Chara australis*; MacRobbie (1962, 1964) on *Nitella translucens*; Barr and Broyer (1964) and Barr (1965) on *Nitella clavata*; Gutknecht (1965, 1966) on *Gracilaria foliifera* and *Valonia ventricosa*; and Dodd, Pitman, and West (1966) on *Chaetomorpha darwinii*.

All these cells have an inside-negative PD across the plasmalemma; all, except *Valonia* and *Chaetomorpha*, also have the vacuole negative with respect to the external solution. The major intracellular cations in all cases are K and Na, with the K/Na ratio in the cells higher than in the external solution. The major intracellular anion is Cl, which is more concentrated in the vacuole than in the external solution. Thus, with the possible exception of *Valonia* and *Chaetomorpha*, there is an inwardly directed Cl pump. It is possible that a Cl pump in the Siphonocladales is short-circuited by a high passive permeability to Cl (Dainty, 1962). The application of the criteria for active transport indicates that in all these cells there is an active Na extrusion. In many cases the distribution of K is in accord with the passive driving forces. An active K influx mechanism is present in *Chaetomorpha*, *Valonia*, and the *Nitella* spp. In *Nitella translucens* (MacRobbie, 1962), the ouabain sensitivity of the K influx suggests the operation of a coupled K-Na pump such as is found in animal cells (Skou, 1964). Dainty (1962) has suggested that such a coupled K-Na pump is present in all algae, but the K component only manifests itself in cells with a low passive permeability to K.

In some of these investigations the light- and temperature-dependence of the ion fluxes have been determined; in these photosynthetic cells, the active fluxes (except Br influx in *Gracilaria* and K influx in *Chaetomorpha*) are light-stimulated; all active fluxes have a high temperature coefficient, as would be expected from their obligate linkage to metabolism. The fluxes down the free energy gradient have variable responses to darkness and low temperature.

All the investigations on freshwater plants mentioned here have been carried out on members of the Characeae. These cenocytes were chosen as experimental material in view of their large size, with consequent ease of separation of cytoplasm, cell wall, and vacuole for chemical and radiochemical analysis, and for electrical measurements at the phase boundaries.

Blinks and Nielsen (1939) pointed out that the genus *Hydrodictyon* shared these advantages of the Characeae. Canny (1956) has shown that these advantages are particularly seen in *H. africanum*. He also pointed out that this species has additional advantages, in that the spherical cenocytes dissociate spontaneously from one another when the cenobium (net) is mature, and that the cenocytes are not connected by plasmodesmata at any stage of their life; also the peculiar morphogenesis of this species produces 256 or 512 ceno-

cytes of identical age and genetic constitution from each zygote. The genus *Hydrodictyon* belongs to the order Chlorococcales (other members of which are the familiar experimental organisms *Chlorella*, *Ankistrodesmus*, and *Scenedesmus*) of the Chlorophyceae. Pocock (1960) has recently reviewed the taxonomy and life history of the genus.

There is some previous work on the ionic relations in *Hydrodictyon* spp., but it is incomplete. Canny (1956) measured ion concentrations and net and tracer fluxes in *H. africanum*, but did not measure the PD. His results indicated that K influx was metabolically mediated, while Na influx was not. Concentrations of K, Na, and Cl in the vacuole and external solution, and the PD between the two phases have been measured in *H. reticulatum* (Janacek, personal communication) and *H. petanaeforme* (Blinks and Nielsen, 1939; Blinks, 1947). Application of the Nernst equation to these data indicates that, if the cells are in flux equilibrium, K and Cl are pumped into the cells, and Na is pumped out.

It was therefore decided to investigate *H. africanum* with respect to the factors influencing the distribution of the major intracellular ions, for a comparison with the Characeae and the marine Chlorophyceae and Rhodophyceae which have been investigated. The aim was to determine which fluxes were active, to compare the effects of light and temperature variation on the various active and passive fluxes, and to investigate the effect of a cardiac glycoside, ouabain, on the cation-regulating system.

MATERIALS AND METHODS

A. Culture Methods

Zygotes of *Hydrodictyon africanum* were kindly provided by Mr. E. A. George from the Cambridge Culture Collection of Algae and Protozoa; they were from the stock used by Canny (1956) and Northcote, Goulding, and Horne (1960). Soil and water culture medium was prepared according to the method given by these authors. The medium had a pH of 7.5–7.8, and contained Na 2.5 mM, K 0.17 mM. Dormant zygotes produced in old cultures germinated in a few days when transferred to a new culture medium. After 4–5 wk in a north-facing window the cenobia had grown to a few millimeters in diameter. The most vigorous of these were then transferred to a new culture solution in a refrigerated tank at 12–14°C. While *H. africanum* will complete its life cycle at 20°C or above, the production and maintenance in a healthy state of cenocytes larger than 1 mm diameter will not occur above 18°C (Canny, 1956). Light was provided for 16 hr a day from two 40 w "warm white" fluorescent tubes and a 60 w tungsten filament lamp, all 50 cm away from the cultures.

Cenobia produced in these cultures have 256–512 cenocytes, and can be up to 9 cm in diameter. At maturity the cenocytes usually dissociate from one another. Growth to a diameter of 6 mm takes about 3 months from germination; under these conditions the life cycle is completed in about 5 months. Cenocytes used in the experiments were 2–4 months old, and were 3–6 mm in diameter.

B. *Tracer Flux Measurements*

Experiments were carried out in a refrigerated water bath, with temperature control to ±0.05°C. Illumination was provided by 2 × 40 w "warm white" fluorescent tubes 45 cm above the experimental material. The energy flux incident on the cenocytes, as determined by a solarimeter of the type described by Monteith (1959), was about $1.4 \cdot 10^4$ erg. cm^{-2} sec^{-1}. The cenocytes were contained in boiling tubes treated with silane. The experimental solution, unless otherwise specified, was the artificial pond water of MacRobbie (1962); i.e., NaCl 1.0 mM KCl 0.1 mM, $CaCl_2$ 0.1 mM, pH about 6.0. The cells were pretreated overnight in this solution before the experiment; they were pretreated for a further 2 hr in the solution to be used in the experiment. Such 2 hr pretreatments were adequate to obtain steady fluxes under all the experimental conditions used.

^{42}K and ^{24}Na were obtained from Atomic Energy Research Establishment Harwell as the spectroscopically pure carbonates, and converted to the chlorides by adding the calculated quantity of HCl. $^{22}NaCl$ and $^{36}NaCl$ were obtained from the Radiochemical Centre, Amersham. These isotopically labeled solutions were substituted for all or part of the corresponding unlabeled salt in the experimental solution.

Isotopic influx was measured by immersing the cenocytes in the labeled solution for a period of 2–6 hr, then removing the cenocytes to an unlabeled solution of 0.6 mM $CaCl_2$ to remove the tracer in the free space. Measurements on isolated cell walls labeled with ^{36}Cl, ^{42}K, or ^{22}Na indicated that 5, 20, and 40 min respectively in this $CaCl_2$ solution (changed at 5 min intervals) were adequate to remove the free space label. The cenocyte diameter was measured using a micrometer eyepiece in a binocular microscope (Canny, 1956). The radioactivity was measured in a 20th Century M6 liquid-counting tube (20th Century Electronics Ltd., New Addington, Croydon, Surrey, England), the cell being wet-ashed in 10 mM HNO_3; or on a planchette under a Mullard MX 123 end window G-M tube (Mullard Ltd., Mitcham Junction, Surrey, England). Liquid counting was not used with ^{36}Cl. Two influxes were measured; M'_1, the vacuolar influx, and M_1, the whole cell influx (see MacRobbie, 1964).

As the uptake of tracer into the cenocytes was linear with time, M'_1 or M_{ov}, is defined as, $\dfrac{Q_v^*}{t \cdot A}$, where Q_v^* is the tracer content of the vacuole, t is the time of the experiment, and A is the surface area of the cell. M_1, or M_{oc}, is defined as $\dfrac{Q_t^*}{t \cdot A}$, where Q_t^* is the tracer content of the nonfree space of the cenocyte.

Q_v^* and Q_t^* are expressed in terms of the specific activity of the external solution, in counts per minute per millimicromole.

Efflux of ^{42}K and ^{22}Na was measured by placing the labeled cenocytes in 10 ml of inactive solution, and replacing this solution (by means of a pipette) at various intervals of time. The 10 ml aliquots were liquid-counted. At the end of the experiment the cenocytes were wet-ashed in 10 ml of 10 mM HNO_3, liquid-counted, and assayed for K and Na by flame photometry. For ^{36}Cl efflux, 1 ml aliquots of inactive solution were used; these were dried down on planchettes and counted under an end window G-M tube. At the end of the experiment the cell was wet-ashed in 0.2 ml of 100 mM HNO_3, electrometrically titrated for Cl content, and then dried down and counted on a planchette.

The efflux, M_{vo}, is defined as

$$\frac{Q_v \cdot k}{A}$$

where Q_v is the vacuolar content of the ion, k is the rate constant for the slow phase of isotopic exchange, and A is the surface area of the cenocyte.

Ouabain was obtained from Sigma Chemical Co. (St. Louis, Mo.), and was made up in a 1 mM stock solution in artificial pond water or some other experimental solution, and diluted as required. A fresh solution was made up every 2 days.

Unless otherwise stated, all experiments were done at 14°C.

C. *Measurements of Photosynthesis*

Photosynthetic rates were measured using $NaHC^{14}O_3$ obtained from the Radiochemical Centre, Amersham. 1 mM $NaHCO_3$ in artificial pond water was labeled with $NaHC^{14}O_3$, and the pH was adjusted to 6.0 with HCl. The specific activity of the external solution was measured with 10 μl samples dried down in 0.1 ml of saturated $Ba(OH)_2$ solution on a planchette. The cells were counted on planchettes after HCl treatment to remove any unfixed $^{14}CO_2$. Calculations showed that self-absorption was negligible.

D. *Chemical Analysis for K, Na, and Cl*

Samples of vacuolar sap for chemical analysis were obtained by cutting open a cenocyte with a scalpel and removing 5 or 10 μl of sap with a microcapillary. This was then put in 10 ml of water for analysis of K and Na, or into 0.2 ml of 100 mM HNO_3 for Cl determination. The rest of the vacuole was then removed with a microcapillary, and the wall and cytoplasm were placed in 1 ml of 100 mM HNO_3 to extract the cations; this was diluted to 10 ml for analysis of K and Na. For analysis of Cl, the cytoplasm and wall were placed in 0.2 ml of 100 mM HNO_3. The free space contribution to the K and Na contents of the wall and cytoplasm fraction was calculated from radioactive washout experiments, and the cytoplasmic content obtained by subtraction. For the analysis of whole cenocytes, the procedure for the residual (wall and cytoplasm) fraction, including the free space correction, was applied.

K and Na were determined in an EEL flame photometer (Evans Electroselenium Ltd., Halstead, Essex, England). The standard solutions were similar to the unknowns both in concentrations of K and Na and in the presence or absence of HNO_3.

Cl determination was by electrometric titration of the type described by Ramsay, Brown, and Croghan (1955).

E. *Electrical Measurements*

Electrical potential differences were measured using the apparatus of Spanswick, similar to that described by Spanswick and Williams (1964), with intracellular glass microelectrodes filled with 3 M KCl solution. The potential difference across the plasmalemma was obtained by inserting a microelectrode through the cell wall till the low cell wall potential suddenly increased. This potential was taken to be the cytoplasmic potential if it decreased suddenly when the electrode was pushed a few

microns further into the cell. This change was equated with penetration of the tonoplast, and the potential with the vacuolar potential, since the lower potential recorded after this further insertion was not altered by pushing the electrode a further 50 μ into the cell. The contention that the cytoplasmic potential referred to above is a true intracellular potential is supported by the finding that it had a transient response to a light-dark change similar to that observed with the vacuolar potential. This transient takes the form, for a light-dark transition, of a depolarization of about 3–4 mv, which occurs within 30 sec, followed by a hyperpolarization to some 8 mv above the normal resting potential, followed by a decline to the normal resting potential in about 5 min. The reverse changes occur in a dark-light transition. Apart from this initial transient, the resting potential of the cells is not significantly altered between light and darkness over the longest period measured (8 hr).

TABLE I

Ion	C_v	C_c	C_o	M_{ov}	M_{vo}	$\dfrac{M_{ov}}{M_{vo}}$ (observed)	$\dfrac{M_{ov}}{M_{vo}}$ (calculated)
K	40±2	93±12	0.1	1.4±0.2	1.5±0.3	0.94	0.09
Na	17±1.5	51±8	1.0	0.7±0.1	1.0±0.2	0.70	2.00
Cl	38±2	58±6	1.3	1.4±0.06	0.6±0.2	2.30	0.001

E_{vo}, the PD between the vacuole and the bathing solution, = -90 ± 4.5 mv.
E_{co}, the PD between the cytoplasm and the bathing solution, = -116 ± 3 mv.
C_v, C_o, C_c = concentration of the ion in millimolar per liter in the vacuole, cytoplasm, and bathing solution respectively. The cytoplasmic concentration involves the use of the measured value of the cytoplasmic thickness of 20 μ.
M_{ov}, M_{vo} = tracer influx and efflux into and out of the vacuole, treating the cytoplasm as a compound membrane, in pmoles cm^{-2} sec^{-1}.

RESULTS

Table I gives values for the concentrations of Na, Cl, and K in the external solution, cytoplasm, and vacuole of *Hydrodictyon africanum*, the tracer influx and tracer efflux of these ions, measured at 14°C in the light, and the electrical potential difference between vacuole and bathing solution, and between cytoplasm and bathing solution. (All errors quoted are standard errors of the mean.)

Canny (1956) has shown that K + Na contribute more than 80% of the total cations in the cell sap of *H. africanum*, so that these ions and Cl are the main intracellular ions. The K/Na ratio is higher in the sap and cytoplasm than in the bathing solution.

The influxes quoted in Table I are those obtained from measurements of the radioactivity of the vacuolar sap; i.e., are M_1' in the terminology of MacRobbie (1964). It has been found from comparisons of specific activities in the cytoplasm and the vacuole of *H. africanum* during a tracer influx experiment that the tonoplast fluxes (M_2) of K, Na, and Cl are much higher than

the plasmalemma fluxes (M_1). As a result of this, and also because the ion content of the cytoplasm is relatively small, $M_1' \approx 90\% \ M_1$ in the 6–8 hr influx experiments quoted in Table I. The conclusion that the fluxes at the tonoplast are much higher than those at the plasmalemma is supported by the finding that the nonfree space of *Hydrodictyon africanum* exchanges, as found in efflux experiments, with a single time constant for K, Na, and Cl. In an efflux experiment the specific activities of the cytoplasm and the vacuole are not significantly different. The rate-determining step in both tracer influx and efflux is at the plasmalemma.

If this system of tonoplast, cytoplasm, and plasmalemma were a single membrane, then C_v, C_o, M_{ov}, M_{vo}, and E_{vo} are related by the equation

$$\log_{10}\left(\frac{M_{ov}}{M_{vo}}\right) = \frac{-zE_{vo}}{58} + \log_{10}\left(\frac{C_o}{C_v}\right) \tag{1}$$

(Ussing, 1949; Teorell, 1949), where $E_{vo} = (E_v - E_o)$ in millivolts. This equation is valid only for passive, independent fluxes, and even in artificial membrane systems it is not obeyed perfectly (Meares and Ussing, 1959). It will be seen from Table I that the measured flux ratios for all three ions are very different from those calculated from equation 1. In the case of Na and Cl the measured flux ratio is such as to show that there is a net ion flux in the direction opposite to that predicted for the passive driving forces, and it may be concluded that Cl is actively transported into the cells in the light, while Na is actively transported out. For K, the data of Table I show that while the observed flux ratio is very different from that predicted by equation 1, it does not show a net transport of K in the direction opposite to that predicted from the passive driving forces. While the data are consistent with active K influx, they are also consistent with a large exchange diffusion component in the K fluxes. The latter is unlikely on the basis of other data (Raven, 1966, and unpublished data), since no experimental treatment causes equal changes in K influx and K efflux. The data presented in Table I were all collected from experiments on cells from the same net; in three out of five other experiments in which K influx and K efflux in the light have been directly compared, a net K influx has been demonstrated.

In an experiment in which M_{ov} for K was 1.7 $\pm$ 0.2 pmoles cm^{-2} sec^{-1}, and M_{vo} was 1.05 $\pm$ 0.11 pmoles cm^{-2} sec^{-1}, the value of E_{vo} was -87 ± 4 mv, and $[K]_v$ was 37 $\pm$ 4 mM. The potential and concentration data give a calculated value for M_{ov}/M_{vo} of 0.1, using equation 1. The observed flux ratio in the light was 1.6. Hence it may be concluded that K is actively transported into these cells in the light, as is Cl, while Na is actively extruded. The energy for this active transport (i.e. net transport against a free energy gradient) most probably comes from metabolism, but it is possible that it comes from linkage to the downhill transport of some other substance.

Since the K/Na ratio in the cytoplasm is much nearer to that in the vacuole than to that in the external solution, it would seem most likely that the K influx and Na efflux pumps are at the plasmalemma.

Figs. 1 and 2 show the effect of low temperature on the influx (M_{ov}) and efflux (M_{vo}) in light of the ions K, Na, and Cl. It will be seen that the fluxes (K and Cl in, Na out) which are partly active are greatly reduced at
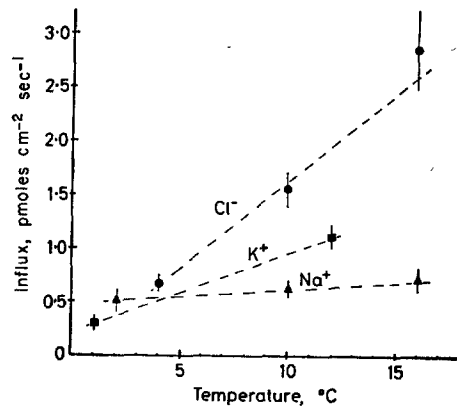

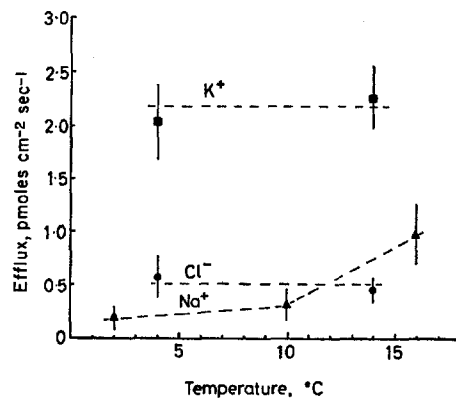Fig. 1. Effect of temperature on the influx of K, Na, and Cl in the light.

Fig. 2. Effect of temperature on the efflux of K, Na, and Cl in the light.

low temperatures. Those which can be explained entirely in terms of passive driving forces (Na influx, K and Cl efflux) are less temperature-sensitive.

Table II shows the values of influx and efflux $(M_{ov}$ and $M_{vo})$ for K, Na and Cl in the light and in the dark in representative experiments. Also shown is the range of dark fluxes, expressed as a per cent of light controls, which have been found. The most light-stimulated fluxes are the Cl influx and K influx; the Na efflux is rather less light-stimulated. Na influx and Cl efflux are often not light-stimulated, while K efflux is rarely more than halved in darkness. A comparison of the values for dark Cl influx and dark Cl efflux in Table II indicates that there is a net Cl influx in these cells in the dark; this influx is

TABLE II

LIGHT EFFECT ON ION INFLUX AND EFFLUX

Flux	Light	Dark	Range of dark fluxes as % light
pmoles cm^{-2} sec^{-1}			
K influx	1.1±0.14	0.14±0.04	10–50
K efflux	1.4±0.17	1.04±0.12	30–90
Cl influx	2.55±0.05	0.9±0.10	10–50
Cl efflux	0.65±0.17	0.61±0.20	60–100
Na influx	0.44±0.02	0.34±0.04	70–100
Na efflux	0.71±0.09	0.31±0.05	20–60

active by the criteria used in evaluating the data of Table I. In many experiments a net efflux of Cl is found in the dark.

Table III gives values for the effect of ouabain (0.5 or 1 mM) on the light influxes of Na and Cl and effluxes of K and Cl, the light and dark K influx and Na efflux, and on photosynthesis. Ouabain at this concentration has no significant effect on any fluxes except K influx and Na efflux. It usually has a slightly stimulatory effect on the other fluxes and on photosynthesis. K influx

TABLE III

EFFECT OF OUABAIN ON ION FLUXES AND PHOTOSYNTHESIS

Fluxes in pmoles cm^{-2} sec^{-1}. Influx = M_{oc}, efflux = M_{vo}. Temperature 14°C unless otherwise stated.

Flux	Control	+ Ouabain
Ouabain 1 mM		
K influx, light	(1) 1.1±0.16	0.72±0.1
	(2) 0.67±0.14	0.17±0.04
K influx, dark	(1) 0.55±0.04	0.37±0.04
	(2) 0.07±0.004	
$^{14}CO_2$ fixation, light, pH 6.0, 1 mM total CO_2	21.6±1.4	27.5±5.7
Cl influx, light	1.75±0.15	2.1±0.25
Na influx, light	0.16±0.026	0.18±0.026

Flux	Control	+ Ouabain
Ouabain 0.5 mM		
K influx, light	1.53±0.18	0.56±0.08
K influx, dark	0.25±0.05	0.29±0.04
Na efflux, light	0.76±0.12	0.21±0.04
Na efflux, dark	0.29±0.06	0.16±0.02
K efflux, light	1.8±0.18	2.3±0.41
Cl efflux, light	2.4±0.16	2.6±0.52
K influx, light 3°C	0.19±0.03	0.17±0.02
Na efflux, light 4°C	0.06±0.01	0.055±0.01

and Na efflux in the light are considerably inhibited, as is Na efflux in the dark. The effect on K influx in the dark is variable; the effect is either insignificant or inhibitory. The ratio ouabain-sensitive K influx/ouabain-sensitive Na efflux is about 1.5 in the light. It varies between 0 and 0.5 in the dark. 0.1

TABLE IV

Na EFFLUX, M_{vo}, PMOLES CM^{-2} SEC^{-1}

Conditions	Experimental solutions	
	NaCl 1 mM CaCl$_2$ 0.1 mM KCl 0.1 mM	NaCl 1 mM CaCl$_2$ 0.1 mM
Light, 14°C	0.44±0.024	0.16±0.02
Dark, 14°C	0.17±0.023	0.165±0.027
Light, 3°C	0.036±0.004	0.038±0.004
Dark, 3°C	0.032±0.005	0.030±0.004

mM ouabain causes an inhibition of K influx and Na efflux of up to 50% of the maximal inhibition, which is reached at 0.5 mM. The fact that in the light K influx and Na efflux are not ouabain-sensitive at low temperature indicates that the ouabain effect is on the active component of the fluxes. These results
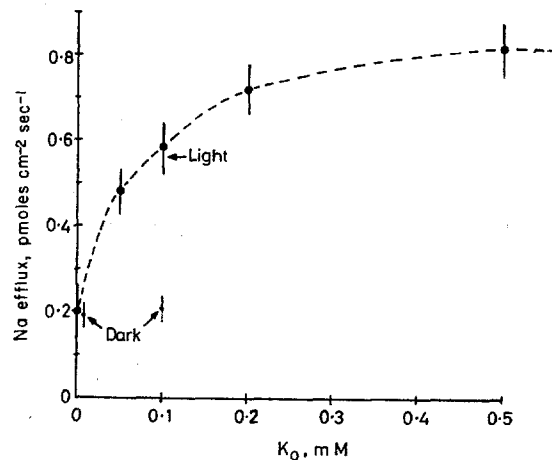

FIGURE 3.   Effect of the concentration of external K on Na efflux in the light and in the dark.

suggest that an ouabain-sensitive coupled K/Na pump is operating, at least in the light.

Table IV shows that, in the light and at high temperature, the Na efflux is partly dependent on the presence of K in the external solution. This K-dependence is not seen in the dark at 14°C, or at 4°C in the light or the dark. This again would suggest a coupling of K influx and Na efflux in the light.

Fig. 3 shows the concentration-dependence of the K effect on Na efflux in the light. A plot of V (the difference between the K-stimulated and K-free Na efflux at a given concentration of K) against V/S gives a value for the apparent K_m. The data of Fig. 3 are replotted in this form as the lower line
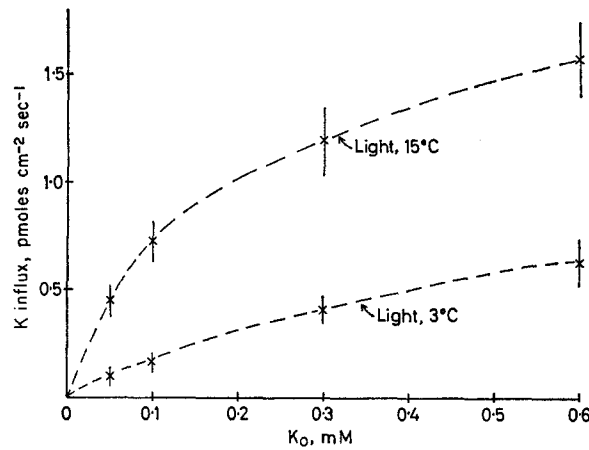

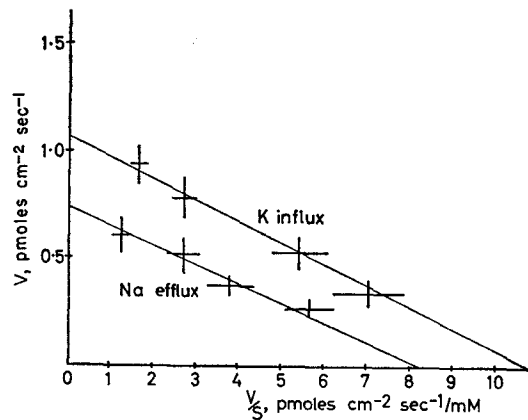FIGURE 4. Effect of the concentration of external K on K influx in the light at 15°C and 3°C.

FIGURE 5. The lower line is V, the difference between the Na efflux at a given concentration of external K ($=S$) and the Na efflux in the absence of external K, plotted against the corresponding value of V/S. The upper line is a similar plot for K influx when V is the difference between the K influx at 15°C and that at 3°C, at various values of S, the external K concentration. The data are respectively those of Figs. 4 and 5.

in Fig. 5. The K_m in this experiment was about 0.08 mM K; other experiments suggest a range of values of the K_m of 0.04–0.08 mM.

Fig. 4 shows the concentration-dependence of K influx at 15°C and at 3°C, measured on the cenocytes from the same net as those that were used in the experiment shown in Fig. 3.

If it is assumed that the passive component of K influx is relatively temperature-independent, like the K efflux, the differences between the two curves are mainly due to the temperature dependence of the active component. This assumption is supported by the finding that the 3°C K influx is not significantly different from the flux at 15°C in the presence of 1 mM ouabain. In the experiment shown in Fig. 5, the K influx at 15°C in the presence of 1 mM ouabain is 0.19 ± 0.03 pmole cm^{-2} sec^{-1} from a solution containing 0.1 mM K, and 0.69 ± 0.11 pmole cm^{-2} sec^{-1} from a solution containing 0.6 mM K. Hence the difference between the two curves will give the dependence of the active K influx on $[K_o]$. A plot of V against V/S for this difference is the upper curve in Fig. 5. The K_m in this experiment is about 0.1 mM K; the range of K_m found in several experiments is 0.03 − 0.1 mM K. These values are similar to those obtained for the dependence of Na efflux on $[K_o]$, and suggest that these two effects of external K are mediated at the same site. Hence K probably stimulates Na efflux via its own active influx.

DISCUSSION

The results shown in Table I suggest that there are active processes moving K and Cl in, and Na out, somewhere between the external solution and the vacuole in *H. africanum*. The further localization of these pumps requires a knowledge of the ion concentrations in that phase of the cytoplasm which is in osmotic equilibrium with the vacuole. On grounds of osmotic equilibrium, this phase, the "ground cytoplasm," cannot have the concentrations given in Table I for the cytoplasm as a whole. It is unlikely that the tonoplast could support the necessary hydrostatic pressure. It would seem likely that many of the ions are in organelles. The cytoplasm of *H. africanum* is more than 50% by volume chloroplasts (Costerton, personal communication). This would be in agreement with the findings of MacRobbie (1962, 1964), Spanswick and Williams (1964), and Kishimoto and Tazawa (1965), who separated chloroplast-containing from chloroplast-free cytoplasm in *Nitella* spp., and determined ion concentrations in these fractions. On grounds of osmotic equilibrium it is unlikely that the [K + Na] in the ground cytoplasm is more than 50% higher than [K + Na] in the vacuole, since the cytoplasmic anions and nonelectrolytes must be accounted for osmotically. Since the measured potential E_{cv} of −26 mv would support, at equilibrium, a three times greater concentration of monovalent cations in the cytoplasm than in the vacuole under the influence of passive forces only, some cation (K or Na or both) must be pumped from cytoplasm to vacuole. The work on *Nitella* spp. quoted previously suggests that the Na concentration in the ground cytoplasm is lower than that in the vacuole, while the K concentration is rather higher. If this is the situation in *H. africanum*, then the measured value of E_{co} would give stronger evidence for an Na extrusion pump than is provided by com-

parison of the observed and calculated flux ratios in Table I. The case for a K influx pump at the plasmalemma stands.

Hence there would seem to be a K influx and Na efflux pump at the plasmalemma, a cation influx pump at the tonoplast, a Cl pump somewhere between the external solution and the vacuole, and a mechanism for ion accumulation in the chloroplasts. A Cl influx pump and Na efflux pump are common to all the species mentioned in the Introduction, with the possible exception of a Cl pump in *Valonia* and *Chaetomorpha*. MacRobbie (1962) has provided good evidence for active K influx in *Nitella translucens*; such a pump is probably present in some of the other species. There would appear to be no K influx pump in *Nitellopsis obtusa* (MacRobbie and Dainty, 1958 *b*) or *Chara australis* (Hope and Walker, 1960; Hope, 1963).

The results shown in Table I, which are taken from experiments done on cells from the same net, show a large unbalanced movement of charge across the membrane. If it is assumed that the tracer flux measurements are valid, then the imbalance of charge must be explained in terms of a net cation influx or a net anion efflux. The net Ca flux measured by Spanswick and Williams (1965) in *Nitella translucens*, while being in the right direction to explain the charge discrepancy, is not quantitatively adequate unless the Ca influx is much larger in *Hydrodictyon* than in *Nitella*. The other alternative, an anion efflux, would presumably involve a metabolically produced anion. This possibility has not been explored.

The finding that the fluxes at the tonoplast are higher than those at the plasmalemma is in agreement with the findings of MacRobbie (1962) for *Nitella translucens* and Hope and Walker (1960) for *Chara australis*. The reverse situation was found by Diamond and Solomon (1959) for *Nitella axillaris* and MacRobbie and Dainty (1958 *b*) for *Nitellopsis obtusa*.

The temperature effects shown in Figs. 1 and 2 are in general agreement with the contention that K and Cl influx and Na efflux are wholly or partly active, and necessarily linked to metabolism, while the reciprocal fluxes are passive. While it is not impossible that a passive membrane permeation process has a high activation energy (e.g. Na influx in *Chara australis*; Hope and Walker, 1960), if it does not have a high Q_{10} it is unlikely to be rate-limited by a metabolic process. An exception would be a light-dependent active transport process under light-limiting conditions.

The active or partly active ion fluxes also tend to be more light-stimulated than the passive fluxes. The question of the mechanism of light stimulation of ion fluxes in *H. africanum* will be dealt with in the following paper (Raven, 1967).

The results on light and temperature sensitivity of K and Na influx in *H. africanum* confirm those of Canny (1956).

Since the electrical PD between the cytoplasm and the external solution in

H. africanum does not change between light and darkness over times longer than those over which the fluxes were measured, the changes in the passive fluxes in darkness must be attributed to changes in permeability rather than driving forces. In the case of the light stimulation of K efflux, however, it is possible that the light effect is due to an increased $[K]_c$ in the light, which would increase M_{co} in the absence of an effect of light on E_{co} or P_K (Hope, Simpson, and Walker, 1966). Most of the ion transport processes, active or passive, which have been investigated in algae, are light-stimulated (Hope and Walker, 1960; MacRobbie and Dainty, 1958 a; MacRobbie, 1962; Gutknecht, 1965). Exceptions are passive K influx in *Chara australis* (Hope, 1963), active K influx in *Chaetomorpha* (Dodd, Pitman, and West, 1966), and active Br influx in *Gracilaria foliifera* (Gutknecht, 1965). Light inhibits I influx in *Gracilaria* (Gutknecht, 1965) and Cl efflux in *Nitella translucens* and *Chara australis* (Hope, Simpson, and Walker, 1966).

The finding that *H. africanum* can, in the light, bring about a net influx of K and of Cl, and a net efflux of Na, with all three of these net fluxes being in the opposite direction to those expected on the basis of passive driving forces, indicates that some portion of each of these fluxes must be linked to an exergonic process. The electrochemical data are not, however, sufficient to predict what fraction of the tracer influx of K and Cl or efflux of Na is dependent on exergonic processes. The effects of darkness and of low temperature discussed above indicate that a large proportion of the tracer K and Cl influx and Na efflux, is linked to metabolic processes, while the corresponding fluxes in the same direction as the passive driving forces are not. Hence a large proportion of the tracer K and Cl influx and Na efflux is of the same nature as the net fluxes of these ions, and is linked to metabolism.

The results given in Table III indicate that the only fluxes which are inhibited by ouabain are K influx and Na efflux. This suggests that a K/Na pump such as has been proposed for many animal cells (Skou, 1964) and *Nitella translucens* (MacRobbie, 1962) is operative in *H. africanum*. The absence of effects on the other ion fluxes (active or passive) or on the probable energy source for light-dependent active ion transport (photosynthesis), or on the K influx or Na efflux in the light at low temperature, indicates a specific effect on the cation pump mechanisms. The concentration of ouabain needed for maximal effect on ion transport is much higher than is needed in *Nitella* or most animal systems. There are, however, reports (Judah and Ahmed, 1963) of 10^{-4} M ouabain being needed for 50% inhibition of K/Na transport and transport ATPase activity. The concentration of ouabain used in the experiments reported here is sufficient to inhibit the K pump, not linked to Na transport, in *Hyalophora cecropia* midgut (Harvey and Nedergaard, 1964), and inorganic phosphate uptake in *Ankistrodesmus braunii* (Simonis and Urbach,

1963). However, the specificity of action indicates that, in *H. africanum*, the action is probably on a linked K/Na pump.

Low concentrations of ouabain inhibit K and Na transport in the same direction in *Allium* epidermis (Brown, Jackson, and Dupoy, 1964), but do not affect K or Rb transport in the other plant systems tested (Hope, 1963; Mengel, 1963; Hodges, 1966; Bonting and Caravaggio, 1966; Dodd, Pitman, and West, 1966). However, in only one of these cases (*Chaetomorpha darwinii*) is the K transport known to be active, and in *Chara australis* it is known to be entirely passive. Cummins, Strand, and Vaughan (1966) have shown that 10^{-4} M ouabain inhibits both the steady-state movement of ^{22}Na through thalli of *Ulva lobata* and *Ulva expansa*, and the light-induced transient increase in this movement. It is not clear how this effect of ouabain is related to an Na efflux pump at the cellular level, since Bonting and Caravaggio (1966) found that 10^{-4} M ouabain had no significant effect on the Na content of *Ulva lactuca* over experimental periods of 5–25 hr.

The effect of removal of external K ions on Na efflux at 14°C in the light (Table IV) shows that the light-dependent portion of the Na efflux is K-dependent. This again indicates a coupling of K transport and Na transport, although K stimulation of Na efflux is known in cases in which the fluxes of the two ions appear to be independent; i.e., not coupled (Essig and Leaf, 1963; Eppley, 1959). However, the values for the apparent K_m for the K effect on active K influx and for the K effect on Na efflux (Figs. 4 and 5) indicate that in *H. africanum* these two effects have similar responses to $[K_o]$, and are probably causally related. These results, and the conclusions drawn from them, are similar to those obtained by Glynn (1956) on human erythrocytes.

Since the removal of external K causes a hyperpolarization of 10–15 mv in *H. africanum*, it might be argued that the K effect on the Na efflux could be due to an effect on the passive Na efflux. This is unlikely, in that removal of external K has no effect on Na efflux in the dark, while the hyperpolarization still occurs. Also the passive Na efflux, judging from the Na efflux at low temperature and assuming the same Q_{10} for passive Na influx, would be too small at 14°C to account for the large effect of removal of external K. The Na efflux at low temperature is insensitive to removal of K in light or dark (Table IV).

It may be concluded that, while a large part of the K influx may be inhibited by ouabain without inhibiting the active Cl influx, the Na efflux in the light depends on simultaneous K influx. The apparent coupling ratio (ouabain-sensitive K influx/ouabain-sensitive Na efflux) is 1.5 in *H. africanum* in the light. This is much higher than the values for animal cells quoted by Keynes (1965). The coupling ratio in *H. africanum* in the dark (0–0.5) is much closer to what is found in animal cells. The lower coupling ratio in the dark is

not simply a function of the lower absolute value of the active cation fluxes, since lowering the active cation fluxes in the light by decreasing the external K concentration does not alter the coupling ratio. Hence the alteration in the coupling ratio appears to be a light effect; the nature of this light effect is unknown.

If it is assumed that the ouabain-insensitive portion of the K influx, and all of the Na influx are passive, then it is possible to calculate values for P_K and P_{Na}, the permeability of the plasmalemma to K and Na, using equation 34 of Briggs, Hope, and Robertson (1961). This equation is:

$$M_{in} = -\frac{zFE_{co}P}{RT} \cdot \frac{c_o}{1 - \exp(zFE/RT)} \qquad (2)$$

where z = the algebraic valency

$\quad\quad\quad F$ = Faraday's constant

$\quad\quad\quad R$ = gas constant

$\quad\quad\quad T$ = absolute temperature

$\quad\quad\quad c_o$ = external concentration

Equations (2) and (3) are not valid in general for passive ion fluxes, but are useful tools for rough comparisons of P between one species and another. Using equation (2), it is found that in the light, $P_K = 9.2 \pm 1.6.10^{-7}$ cm sec^{-1}, and $P_{Na} = 7.3 \pm 1.6.10^{-8}$ cm sec^{-1}. In the dark, $P_K = 6.9 \pm 1.3.10^{-7}$ cm sec^{-1}, and $P_{Na} = 6.8 \pm 1.8.10^{-8}$ cm sec^{-1}. These values are similar to those quoted for other freshwater algal cenocytes in the papers mentioned in the Introduction. The value for α (P_{Na}/P_K) of 0.1 for H. africanum is also similar to that found in other algal cenocytes. The ability to demonstrate an active K influx in these algae does not appear to be related to a low value of P_K (Dainty, 1962).

Rough estimates of P_{Cl} were calculated, using equation 35 of Briggs, Hope, and Robertson (1961). This equation is:

$$M_{Cl} = -\frac{FE_{co}P_{Cl}}{RT} \cdot \frac{Cl_c \exp(-FE/RT)}{1 - \exp(-FE/RT)} \qquad (3)$$

A difficulty arises in the use of this equation in that the value of $[Cl]_c$ in the phase of the cytoplasm in contact with the plasmalemma is not known. The assumption has been made, by analogy with the findings of Spanswick and Williams (1964), and of Kishimoto and Tazawa (1965) for Nitella spp., that Cl is in flux equilibrium across the tonoplast, and $[Cl]_c$ has consequently been estimated from $[Cl]_v$ and E_{vc}, using the Nernst equation. Inserting values for the Cl efflux, M_{Cl}, $[Cl]_c$, and E_{co}, a value for P_{Cl} of 7.10^{-9} cm sec^{-1} is obtained in both light and dark. This contrasts with the finding of Hope, Simpson, and Walker (1966) that P_{Cl} in Nitella translucens and Chara australis is higher in the dark than in the light.

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