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IONIC COMPOSITION OF THE CYTOPLASM OF *NITELLA FLEXILIS*[1]

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The K, Na and Cl concentrations of the chloroplast layer and the flowing cytoplasm of *Nitella flexilis* have been determined by applying an internal perfusion technique, which enabled us to avoid contamination of ions from the cell sap. K, Na and Cl concentrations of the chloroplast layer are 110, 26 and 136 mM and those of the flowing cytoplasm are 125, 5 and 36 mM respectively. The cell sap contains 80 mM K, 28 mM Na and 136 mM Cl. Although there are some variations in these values among samples, the flowing cytoplasm is rich in K and poor in Cl and especially in Na. The exchange of K and Na across the tonoplast occurs fairly easily (half-time, a few hours), while that of Cl occurs extremely slowly (half-time, a few days).

In a previous paper (*1*) it is reported that the membrane potential of *Nitella* is composed of the potentials at cell wall, across plasmalemma and tonoplast all in series. Among these the potential across tonoplast is about 15 mv or less (cytoplasm negative to vacuole) (*2*, *3*). The potential at the cell wall is almost zero in 100 mM KCl, but it reaches as high as -90 mv against external solution such as 0.01 mM KCl (*1*). The only excitable membrane is the plasmalemma (*1*). FINDLEY and HOPE (*3*) reported a slow potential change across tonoplast, which was observed only when plasmalemma elicited an action potential.

The exchange of the cell sap with some artificial solutions such as KCl, NaCl, LiCl, RbCl, K_2SO_4, KNO_3, Na_2SO_4 or $NaNO_3$ had only minor influence on the resting and action potentials of *Nitella* (*4*). This result appears to be contradictory to an idea that the resting potential of *Nitella* is simply determined by the ratio of K concentration of cell sap to that of outside medium (*5*) or that the height of the action potential by the ratio of Cl concentration of the cell sap to that of external medium (*6-8*).

It is desirable, therefore, to determine exactly the ionic composition of the cell wall, chloroplast layer, the layer of the flowing cytoplasm and cell sap separately. DAINTY and HOPE (*9*) estimated the concentration of indiffusible anions in the cell wall to be 0.6 equivalent/liter. HOLM-JENSEN et al. (*10*) first measured the ionic content of the cytoplasm of *Nitella sp.* and

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Tolypellopsis. They found no appreciable differences in K and Na concentration between cytoplasm including chloroplasts and cell sap. They, however, noticed a possibility of the error caused by contamination of cell sap into the cytoplasm to be measured. MACROBBIE (*11, 12*) reported that both K and Na concentrations in the flowing cytoplasm of *Nitella translucens* are about 1.5 times larger than that in the cell sap, while Cl concentration is about one-third of that in the cell sap. On the other hand, the coloroplast layer contained about 4–5 times as concentrated K and Na, and about 1.5 times as concentrated Cl as in the cell sap. SPANSWICK et al. (*2*) obtained almost similar results on the flowing cytoplasm except for much lower value for Na.

In this report we introduce another method developed with an attempt to avoid contamination of the cell sap in the analysis of the ionic concentration of the cytoplasm. The changes in ionic concentration of the cytoplasm accompanying artificial modification of the ionic composition of the cell sap will also be described.

MATERIAL AND METHODS

Internodal cells of *Nitella flexilis* were used throughout the experiment. Internodes of 4–7 cm in length were isolated from adjacent cells and were maintained in an artificial pond water[2] for several days before the experiments. The composition of the simplified artificial pond water is 0.1 mM KCl + 0.2 mM NaCl + 0.5 mM $CaCl_2$.

Before analysis of concentrations of K, Na and Cl the internodes were washed at least 3 times by a large volume of deionized water. During this procedure free ions which had been involved in the cell wall were almost completely washed away. It cannot be denied that some ions adhere tightly to the microstructure of the cell wall and hardly be washed away by deionized water. However, the error was proved to be negligibly small.

Ionic concentration of cell sap

Each internode was transferred into polyethylene tubes, each of which contained 3 ml of deionized water, and was centrifuged at 2,300 *g* for 10 minutes. By this procedure the internode was killed and almost all free ions inside the cell leaked out into the outside 3 ml of the deionized water. It is possible to calculate the ionic concentration of the cell sap from the ionic concentration in the 3 ml solution, if we know the sum of the volumes of the cytoplasm and the vacuole. The volume of the internode was calculated from outer diameter and length of the internode. From this the

[2] Composition of the standard artificial pond water is as follows: 2.5×10^{-5} M KCl + 5×10^{-5} M KNO_3 + 2×10^{-4} M NaCl + 2.5×10^{-4} M $CaCl_2$ + 2.5×10^{-4} M $Ca(NO_3)_2$ + 10^{-4} M $MgSO_4$. For convenience, the tap water of Osaka City of which dissolved chlorine gas had been removed by passing through a column of active carbon powder, was frequently used for culturing the *Nitella*. The K, Na, Cl and Ca concentrations were 5.2×10^{-5} M, 2.8×10^{-4} M, 3×10^{-5} M and 1.5×10^{-4} M respectively.

volume of the cell wall was subtracted, which was calculated from the thickness of the layer. Since the ionic concentration of the cytoplasm is not much different from that of cell sap and the volume of the former is only about one-tenth of the latter, the error involved in this simplified method for determination of ionic concentration of cell sap is less than a few per cent.

Ionic concentration of chloroplast layer + flowing cytoplasm

After washing the internode in the deionized water for several times vacuole of the cell was perfused usually with isotonic mannitol (0.3 M) or other isotonic salt solutions containing only foreign ions usually at a rate of 25 μliters/min. The method of exchange of cell sap without harm on the internode has been described previously (*13*, *4*). This procedure seems to be inevitable to avoid contamination of ions from cell sap. After ligating both ends of the perfused internode the sample was transferred into a polyethylene tube containing 3 ml of deionized water and was centrifuged at 2,000 *g* for 10 minutes. Since the chloroplast layer and flowing cytoplasm were driven in a few minutes to the centrifugal end, the volumes of these two cytoplasms could be measured under a microscope. This rate of centrifugation is high enough to kill the cytoplasm sooner or later. Therefore, almost all free ions are expected to be lost into the external solution. The ionic concentrations of these two layers were calculated from the concentrations of ions which had leaked out into the 3 ml water. The samples which showed any deformation of chloroplast during internal perfusion were discarded.

Ionic concentration of chloroplast layer

If the vacuole of the internode was perfused with 0.3 M mannitol or liquid paraffin rapidly, i.e. at a rate 25 μliters/sec, almost all flowing cytoplasm was lost with the perfusing solution, enabling us to measure the ionic concentration in the chloroplast layer with the same procedure described in the preceding paragraph.

Ionic concentration of flowing cytoplasm

The ionic concentration of the flowing cytoplasm can be calculated from that of chloroplast layer + flowing cytoplasm and that of chloroplast layer alone knowing the exact volumes of the chloroplast layer and the flowing cytoplasm.

In other experiments the Na and Cl concentrations of the flowing cytoplasm were measured directly on 20 samples. The cell sap of the internode was first exchanged for 150 mM KNO_3+10 mM $Ca(NO_3)_2$. Then, the sample was centrifuged at 200 *g* for 10 minutes. The internode was ligated with a strip of silk thread at the centrifugal end where the flowing cytoplasm was collected into a column a few mm long. It included tiny vacuoles containing only K, NO_3 and Ca. The presence of these vacuoles, however,

did not cause any error in determining Na and Cl contents of the flowing cytoplasm. Three or four of these samples, the volumes of which were measured under a microscope, were transferred quickly into 3 ml of deionized water and crushed by a forceps before their Na and Cl concentrations were measured.

K and Na concentrations were determined by flame photometry and Cl concentration by colorimetry which is a modified SCHALES and SCHALES' method (*13, 14*). A volume of 3 ml was large enough to measure the concentrations of K, Na and Cl. Therefore, the determination of K, Na and Cl could be performed on a single internode. The values given in the following are the averages on single internodes. These are shown with standard error of mean. The number of the internodes measured is added in brackets at the end of each average.

RESULTS

Volumes of cell wall, chloroplast layer and of flowing cytoplasm

The thickness of the cell wall of mature internode is about 10 μ, which corresponds to about 9 per cent of total cell volume (Table I). The volumes of the chloroplast layer and of flowing cytoplasm are about 5 and 3 per cent of the total cell volume respectively. These values are of mature internodes.

TABLE I

Volumes of the cell wall, chloroplast layer and flowing cytoplasm of the mature internode of Nitella flexilis

The average is shown with the standard error of mean. Number in the brackets is the number of materials measured.

	Thickness of the layer (in microns)	Relative volume of the layer (in per cent)	
Cell wall	8.30±0.22 (10)	6.87±0.21 (10)	July, '64
	10.56±0.54 (9)	8.62±0.20 (9)	July, '63
	10.00±0.32 (10)	9.00±0.30 (10)	July, '63
Chloroplast layer		5.84±0.24 (16)	Oct., '64
		5.13±0.78 (5)	July, '64
		4.63±0.23 (10)	Dec., '64
		6.12±0.55 (5)	July, '64
Flowing cytoplasm		3.18±0.86 (5)	July, '64
		3.33±0.50 (5)	July, '64

Ionic concentration of cell sap

K, Na and Cl concentrations of the cell sap of mature internode are about 80, 28 and 136 mM respectively (Table II). These values varied more or less among samples of different culture condition, age and season.

Ionic concentration of chloroplast layer + flowing cytoplasm

TABLE II

Ionic concentrations of cell sap of Nitella flexilis

K (mM)	Na (mM)	Cl (mM)	
80.2± 4.2 (6)	28.6±5.1 (6)	134.0± 2.6 (3)	Oct., '64
92.3± 3.3 (5)	26.4±4.1 (5)	131.0±18.5 (5)	July, '64
72.8± 3.6 (4)	45.1±2.1 (4)		Oct., '64
94.5±14.6 (5)	42.1±7.4 (5)		Oct., '64
71.5± 4.7 (10)	31.9±2.1 (10)		Oct., '62
82.5± 4.3 (10)	12.1±0.9 (10)	147.0± 6.0 (5)	June, '63
68.2±10.5 (3)	15.4±1.7 (3)	126.5± 9.6 (3)	Oct., '63
80.3±1.73 (43)	27.5±0.7 (43)	135.5± 2.3 (16)	Averages of whole experiments

K, Na and Cl concentrations of these layers are 119, 13 and 82 mM respectively (Table III). The ionic concentrations in the chloroplast layer and in the flowing cytoplasm do not involve ions tightly bound to the structure of the cytoplasm, which could be extracted only by drastic methods such as treating with strong acids.

TABLE III

Ionic concentrations of chloroplast layer + flowing cytoplasm of Nitella flexilis

K (mM)	Na (mM)	Cl (mM)	
81.6± 4.9(8)	6.7±5.4(8)	87.0±3.9 (8)	July, '64
152.0±16.0(10)	14.7±2.3(10)	78.5±5.6 (10)	July, '64
110.0± 7.3(4)	20.8±1.3(4)	78.6±3.6 (4)	July, '64
118.5± 3.9(22)	12.9±1.1(22)	81.6±2.4 (22)	Averages of whole experiments

Ionic concentration of chloroplast layer

K, Na and Cl concentrations of the chloroplast layer of mature internode are about 110, 26 and 136 mM respectively (Table IV). These values are probably of free ions in the chloroplasts.

TABLE IV

Ionic concentrations of chloroplast layer of Nitella flexilis

K (mM)	Na (mM)	Cl (mM)	
108.6± 5.7 (5)	12.1±3.2 (5)	123.2± 8.3(5)	Oct., '64
117.0± 5.7 (10)	35.0±4.2 (10)	161.5±14.6(9)	July, '64
84.3± 3.3 (5)	24.0±4.1 (5)	119.0±18.5(5)	July, '64
121.0±13.5 (6)	23.8±6.1 (6)	122.0±14.6(6)	July, '64
110.1± 2.5 (26)	25.9±2.0 (26)	135.9± 6.1(25)	Averages of whole experiments

Ionic concentration of flowing cytoplasm

K concentration of the flowing cytoplasm is calculated from the data in Tables III and IV as 93–132 mM (*1* and *2* in Table V), which is more or less larger than that of the cell sap (cf. Table II). On the other hand, the concentrations of Na and Cl are certainly much lower than those of the cell sap, though these values vary considerably among samples (*1–4* in Table

TABLE V

Ionic concentrations of flowing cytoplasm of Nitella flexilis

	Calculations	K(mM)	Na(mM)	Cl(mM)	
	Chloroplast layer + flowing cytoplasm	110±7.3(4)	20.8±1.3(4)	78.6±3.6(4)	July, 20, '64
	Chloroplast layer	121±13.5(6)	23.8±6.1(6)	122.0±14.6(6)	July, 20, '64
1)	Flowing cytoplasm	93.0	16.1	12.0	
	Chloroplast layer+ flowing cytoplasm	118 (22)	12.9 (22)	81.6 (22)	Averages of the values
	Chloroplast layer	110 (26)	25.9 (26)	136 (26)	listed in Tables III and
2)	Flowing cytoplasm	132	(-7)	(-2)	IV.
	Flowing cytoplasm	125	2.8	2.0	Averages of 1) and 2)
	Chloroplast layer+ flowing cytoplasm		5.7±1.6(4)	45.0±4.1(4)	Feb. 18, '65
	Chloroplast layer		16.5±9.5(3)	59.1±3.3(3)	Feb. 18, '65
3)	Flowing cytoplasm		2.9	21.1	
	Chloroplast layer + flowing cytoplasm		14.2±4.8(3)	51.8±2.7(3)	Feb. 18, '65
	Chloroplast layer		11.9±2.2(2)	65.6±10.4(2)	Feb. 18, '65
4)	Flowing cytoplasm		18.2	38.0	
	Flowing cytoplasm		9.1	28.2	Averages of 3) and 4)
Direct measurements					
5)	Flowing cytoplasm (cell sap perfused with KNO_3)		4.9±1.5(9)	35.9±9.1(9)	Feb. 20, '65
6)	Flowing cytoplasm (cell sap unperfused)		6.9±0.2(6)	95.2±13.1(6)	Feb. 20, '65

1) The internodes were taken from one batch.

2) The averages are on the internodes of different batch and date. The negative values of Na and Cl concentrations are, therefore, artificial ones. These should be regarded as some small positive values.

3), *4)*, *5)* and *6)* The averages are on the internodes from the same batch. In order to increase the accuracy of measurements 3–4 samples were immersed in the deionized water in one test tube. See text for the details.

1–4 are calculated from the concentrations in the chloroplast layer and those in chloroplast layer + flowing cytoplasm. *5* are the average of the direct measurements on the flowing cytoplasm of the internode whose cell sap was perfused primarily with 150 mM KNO_3 + 10 mM $Ca(NO_3)_2$. *6* are the average of the direct measurements on the flowing cytoplasm of the normal internode.

V). It is desirable to confirm this situation by measuring the ionic concentrations directly on the flowing cytoplasm. Actually the measurements were done on the cells which were full of the flowing cytoplasm and had no central vacuole. According to this method Na and Cl concentrations are about 5 mM and 36 mM respectively on the cell primarily perfused with 150 mM KNO_3 + 10 mM $Ca(NO_3)_2$ (*5* in Table V) and 7 mM and 95 mM on the unperfused cells (*6* in Table V). It is very likely that the larger values of the Na and especially of Cl concentrations listed in (*6*) are caused by the contamination of the natural cell sap. Therefore, it can be concluded that the flowing cytoplasm contains only about one-seventh Na and one-fourth Cl of the cell sap.

Ionic concentration of cytoplasm of internodes having artificial cell sap

The cell sap of normal internode was exchanged for an artificial cell sap containing 150 mM $NaNO_3$ + 10 mM $Ca(NO_3)_2$. After the exchange the sample was kept in 1.5 mM $NaNO_3$ + 0.1 mM $Ca(NO_3)_2$ for hours. The analysis of K, Na and Cl contents of chloroplast layer + flowing cytoplasm were made on this material by applying the same procedure described in the foregoing. The results are shown in Table VI. Referring to Table III it

TABLE VI

Ionic concentrations of the chloroplast layer + flowing cytoplasm of Nitella *internode having artificial cell sap*

Artificial cell sap	Period of incubation (hr)	K (mM)	Na (mM)	Cl (mM)	
150 mM $NaNO_3$ + 10 mM $Ca(NO_3)_2$	2–4	21.7±3.5(11)	76.6±8.2(11)	118.5±10.5(11)	Oct., '64
150 mM NaCl + 10 mM $CaCl_2$	3–5	32 ±5.6(4)	140 ±8.8(4)		Oct., '63

is obvious that during 2–4 hours incubation pepiod K content in the cytoplasm decreased markedly and Na content increased correspondingly, while Cl content did not decrease appreciably. The result shows that an equivalent amount of K has been exchanged for Na across tonoplast fairly easily. On the other hand, only minor amount of Cl was exchanged for NO_3 in the vacuole during a few hours period. This situation was confirmed by another experiment using 150 mM NaCl + 10 mM $CaCl_2$ (Table VI) for artificial cell sap. It is expected that the half time for the exchange of Cl across tonoplast was of the order of days.

DISCUSSION

The concentration of K in the external medium of *Nitella* internode is much smaller than those of Na, Ca and Cl. However, the internode accumulates much ions in the cell sap in such a manner as the concentrations

of K and Cl are much larger than those of Na or Ca. The sum of the concentrations of K, Na and Cl of the cell sap is generally slightly less than the osmotic value of the internode measured in sucrose equivalent. This may be due to the existence of some other inorganic and organic ions which have not been measured here.

The K content in the flowing cytoplasm of the normal internode is of the same order as or slightly higher than that in the cell sap, while the Na and Cl contents are only a fraction of the latter. Although the *Nitella*

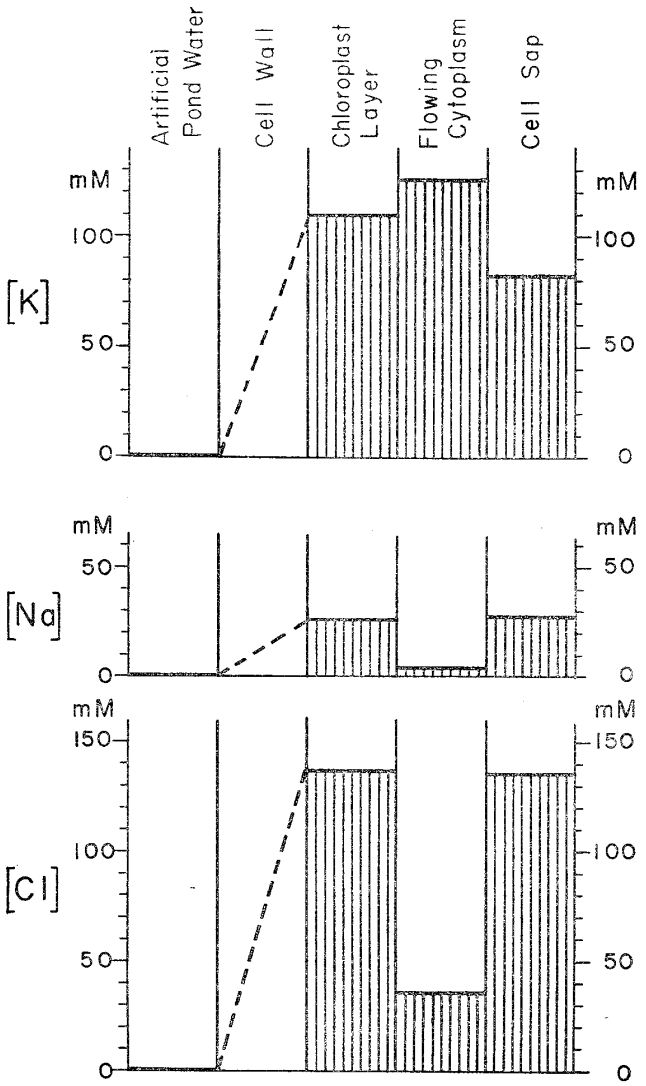

Fig. 1. Concentrations of K, Na and Cl in artificial pond water, chloroplast layer, flowing cytoplasm and in cell sap are shown in diagram. The concentrations in the cell wall phase were not measured here and, therefore, expressed with dashed lines in the figure.

internode used here contains a fairly small amount of Na in the cell sap comparing with the results reported previously (*15, 10, 6*), our result indicates that Na concentration of the flowing cytoplasm is very small like many animal cells (*16*). SPANSWICK and WILLIAMS (*2*) also found a less Na content in the flowing cytoplasm than in the cell sap on *Nitella translucens*. However, according to them more or less larger amount of Cl (65 mM) is contained in the flowing cytoplasm. They measured the Na and Cl contents directly on the endoplasm which was collected from the centrifuged cell. It is likely that the endoplasm thus centrifuged involved many tiny dispersed vacuoles which were rich in Cl. According to our result the Na and Cl concentrations on the unperfused cells are certainly larger than those of the cells primarily perfused with solution containing only foreign ions.

The small concentration difference in K between the flowing cytoplasm and the cell sap is in favor of the small potential difference across the tonoplast (*2, 3, 8*), if the potential is assumed to be a K-potential. According to SPANSWICK and WILLIAMS (*2*) K concentration of the flowing cytoplasm is slightly higher than that of the cell sap and this result is in accord with the tonoplast potential (i.e., 10 mv) measured by them and by FINDLAY and HOPE (*3*) on *Nittela translucens* The sum of the concentrations of K and Na in the flowing cytoplasm is much larger the concentration of Cl, indicating that the electroneutrality is maintained largely by organic anions in the cytoplasm.

Na and especially Cl concentrations in the chloroplast layer are larger than those in the flowing cytoplasm. Such a large content of Cl in the chloroplast layer seems to indicate an active role of the chloroplast in accumulating Cl with Na and/or K in the cell. With careful observations by a phase contrast microscope with oil immersion objective it was found that the chloroplast layer consists of many lines of chloroplasts surrounded by thin film of gel jacket and thin strands connecting the chloroplasts (*17–19*) and that mitochondria of irregular shape attached to the inside of the cell wall (*19*). Many tiny particles were observed to move with ordinary cytoplasmic flow or somewhat irregularly at the same depth as the chloroplasts. The thickness of the so-called plasmagel layer seems to be less than 1 μ. Chloroplast of about 6 μ in long axis of a flat ellipsoid certainly occupies a major volume of this layer. Anyway, it was not intended here to decide whether the ion concentration of this layer is mostly of chloroplast or not.

The K, Na and Cl concentrations of each phase are shown diagrammatically in Fig. 1. It is worthy to note that the concentration profiles of Na and Cl are almost in parallel. Now, by using these values of ionic concentrations the equilibrium potentials of $K^+(E_K)$, $Na^+(E_{Na})$ and $Cl^-(E_{Cl})$ in the chloroprast layer, flowing cytoplasm and vacuole can be calculated, the results of which are shown in Fig. 2. The full line in the Chloroplast Layer is the equilibrium potential of ions calculated on an assumption that the ionic concentration of the layer is the same as that of the flowing cyto-

plasm. However, if the ionic concentration of the chloroplast layer measured is assumed to be of an uniform distribution in the layer, the equilibrium potential is different from above, the result of which is shown with dotted lines in Fig. 2. Heavy dashed dot lines (E) are the potentials actually measured (*1*).

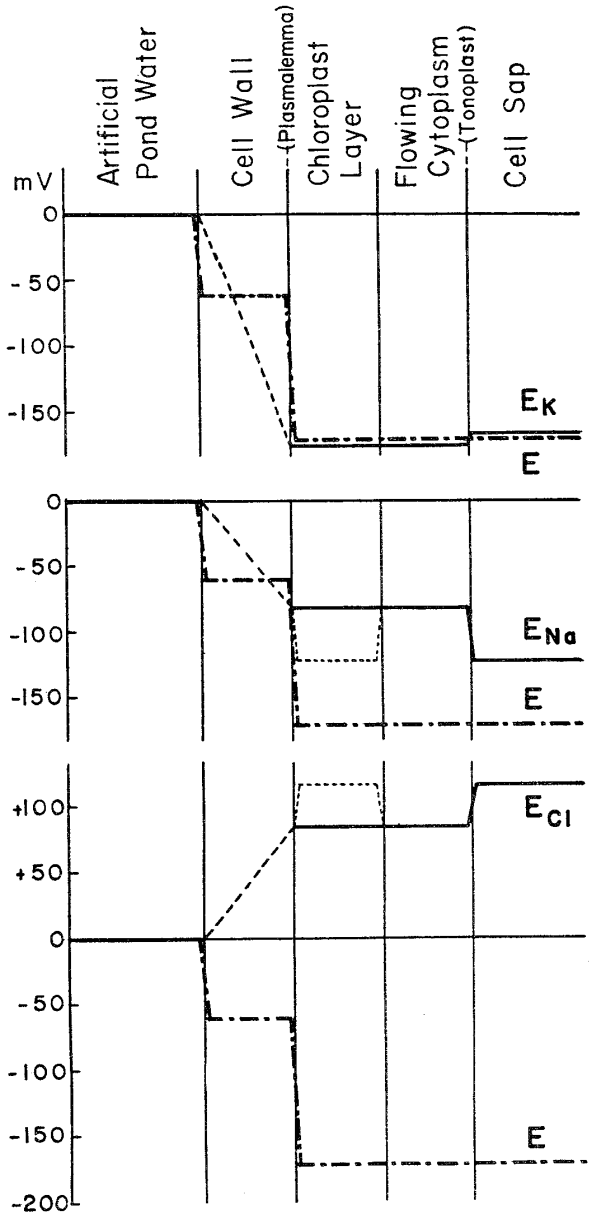

Fig. 2. Equilibrium potentials for K^+ (E_K), Na^+ (E_{Na}) and Cl^- (E_{Cl}) of chloroplast layer, flowing cytoplasm and cell sap against external medium (0.1 mM KCl+0.2 mM NaCl+0.5 mM $CaCl_2$). These are calculated on the basis of NERNST's equation for diffusion potential by using the ionic concentrations in each phase which are shown in Fig. 1.

According to these figures K^+ is almost in electrochemical equilibrium in each phase. A jump in E_k (8 mv) across the tonoplast is of the same order as that actually measured by SPANSWICK and WILLIAM (*2*) and FINDLAY and HOPE (*3*). Electrochemically the equilibrium potential for Na^+ in each phase is less negative comparing with the potential actually measured, which implies an over-all tendency of Na^+ movement towards the vacuole. The concentration profile of Na^+ in Fig. 1 suggests the existence of some active process which may exclude the Na^+ of the flowing cytoplasm both in the direction to external medium and to vacuole. This result is in accord with that previously reported by SPANSWICK and WILLIAMS (*2*) on *Nitella translucens*. On *Nitellopsis obtusa* MACROBBIE and DAINTY (*20*) and on *Nitella translucens* MACROBBIE (*11*) supposed the location of active exclusion of Na^+ at the plasmalemma.

With regard to Cl^- the equilibrium potentials are all positive values, which implies an over-all tendency of Cl^- to move towards external medium. The concentration profile of Cl^- in Fig. 1 suggests the existence of some active process which drives Cl^- towards cell interior both at the plasmalemma and at the tonoplast. MACROBBIE and DAINTY (*20*) reported on *Nitellopsis obtusa*, a brackish water Characeae, that the site of the active Cl-influx locates only at the tonoplast. On the other hand, on *Nitella translucens*, a fresh water species, MACROBBIE (*12*) and SPANSWICK and WILLIAMS (*2*) obtained the results indicating the location of active Cl-influx at the plasmalemma. According to the latter authors Cl^- is in equilibrium across the tonoplast. On the other hand, MACROBBIE did not deny a possibility of the existence of active Cl-influx at the tonoplast.

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