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Uptake and Loss of Na^+, Rb^+, and Cs^+ in Relation to an Active Mechanism for Extrusion of Na^+ in Scenedesmus[1]

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Summary. The mechanism for extrusion of Na^+ from *Scenedesmus* cells is characterized physiologically. It is stimulated by phosphate but oxygen is not necessary. Rb^+ and Cs^+ may also be extruded, but in the presence of Na^+ they cannot compete for the sites on the inside of the transport system. When Na^+ is extruded, Rb^+ and, by inference, K^+ seems to be transported as counter ion from the outside, and sodium ions compete only weakly for this external site. The parallelism between these findings and the Na^+-K^+-activated adenosine triphosphatases known from animal tissues is pointed out.

With low additions of phosphate, the extrusion mechanism can keep the cells practically free from Na^+. Increasing the concentrations of external phosphate stimulates uptake more than extrusion, and a net uptake occurs. As for Rb^+ and Cs^+, they are taken up in the absence of external phosphate, but additions of P will greatly enhance the amounts absorbed. Two different ways of uptake are indicated.

Extrusion of sodium is well known from animal cells and tissues (17). In plants, the phenomenon has been studied in species adapted to salt or brackish water, but little evidence has been available from freshwater forms (2, 12). The observations of Rathje (13) on yeast, *Chlorella,* and *Lemna* are an exception, although they were discussed in other terms (14). Biochemical (17) and biophysical (2) mechanisms have been suggested, but it is less clear how they would fit in a physiological system. Simonis and Urbach (15) noted an effect of Na^+ on phosphorylation in *Ankistrodesmus,* although they did not directly correlate their observation with a mechanism for extrusion of sodium ions.

During work at Wageningen, The Netherlands, the author had occasion to develop cultures of *Scenedesmus* with advanced phosphorus deficiency and to study the reaction of these inhibited cells to additions of phosphate (11). The results permitted a systematic study of the way in which the uptake and loss of different ions may be affected by control alterations in phosphate metabolism of cells without the use of exogenous inhibitors. The approach led to physiological evidence for the action of an active sodium extrusion mechanism in this fresh-water alga (12). The present paper gives more detailed data regarding this extrusion mechanism, which also affects the movements of univalent cations other than Na^+. Actually, we have a physiological counterpart to the enzyme systems isolated from animal tissues by Hokin and Hokin (7), Jarnefelt (8), Skou (16, 17), and others, and suggested by them to be the biochemical basis for sodium transport. Consideration will also be given to the relationship with some biophysical data and with studies on competition between the ions of the alkali metals.

Materials and Methods

The same strain of *Scenedesmus spec.* was used as in earlier work (11), and the P-deficient cells developed according to the same method, with only minor modifications. Thus, the light intensity was approximately 15,000 ergs per second and cm^2, and in addition to the Na citrate, 0.15 mM Na_2/EDTA was used to chelate the iron. It is pertinent to the present problem that the standard nutrient solutions are low in sodium, and contain only 0.625 meq/liter.

Further, the P-deficient cultures proved sensitive towards air pollution, and ceased growth long before any ill effects could be noted in the precultures with +P conditions. This trouble could be overcome by inserting a filter with active carbon in order to purify the gas mixture supplied (air + 5 % CO_2). Cells with a P content of only 0.2 to 0.3 mg/g fresh weight as against 3 to 5 mg/g fresh weight in normal cells could then be produced, which corresponds to the degree of deficiency obtained earlier (11).

The experiments presented were normally performed at the light intensity mentioned above. Darkness made quantitative but no qualitative differences.

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The data on the effects of anaerobiosis were per force collected in darkness.

The approximate weight of the cells in a culture could be estimated from the light absorbancy at 525 mμ. With the aid of the figure obtained a final cell suspension was prepared, after 2 centrifugations and rinsings in glass-distilled water, so that aliquots containing about 5 mg dry weight could be given to each vessel of an experiment. The exact weight of the algae used was determined in separate aliquots (cf. 20).

The experimental vessels consisted of 50 ml Erlenmeyer flasks kept at 25° in a shaking water bath adjusted to about 90 cycles per minute. The flasks were open to the air, or the bath was covered with a hood and gassed with a stream of nitrogen. In the latter case, the experimental solutions were shaken in the anaerobic conditions for 3 hours before the cells were added, so as to minimize errors due to remnants of dissolved O_2.

Each flask contained 10 ml of algal suspension with 25 mM KNO_3 and 1 mM $MgSO_4$. The solutions were kept at pH 6.4 to 6.5, in the first experiments by Na citrate buffers with a concentration of 27.5 mM. The phosphate was in these cases supplied as a mixture of K-Na-phosphates at a pH of 6.5, with the initial phosphate concentration varied as stated in each case. Additions of a KCl-NaCl mixture were made so as to keep K^+ and Na^+ constant. When the need for solutions low in sodium developed, K-citrate, K-phosphates and KCl were used in a corresponding way.

The ions $^{22}Na^+$, $^{86}Rb^+$, or $^{137}Cs^+$ were given at 10 μc per liter. In order to give a well-defined medium, 1 mM of the corresponding, unlabeled chloride was always present, both in the uptake and in the outflow studies.

At the end of the uptake experiments, the cells were spun down twice in a centrifuge and resuspended in the corresponding, inactive and phosphate-free solution, so as to avoid interference from ions in the free space. They were then filtered on glass fiber filters, washed once more with the same solution as used for resuspension, and, finally, with distilled water. After the outflow experiments, the cells could be filtered off directly and washed on the filter, twice with medium and twice with distilled water. The preparations were glued to aluminum planchets, dried at 60°, and brought to the counter.

The radioactivity was calculated as μeq/g dry weight by reference to standards prepared from the original solution. The lowest quantity determinable with any accuracy under the conditions used was in the order of 0.1 μeq/g dry weight. This limitation became relevant only for the experiments with sodium uptake. The dry weight of the cells is about 30 % of their fresh weight.

Each treatment within an experiment was run in duplicate flasks, the single values differing less than 10 % from the averages given in the tables. Experiments of the same kind were regularly performed on separate days and with separate batches of cells. Such series give qualitatively the same results, whereas quantitatively they may differ by 20 to 30 %.

Results

Influence of Phosphate Additions on the Uptake of Alkali Ions. Rb^+ and Cs^+ are taken up in measurable quantities by the P-deficient cells without any external additions of phosphate. However, such additions increase the uptake, and the amount taken up also increases with the time of the experiment (table I).

Table I. *Uptake of Na^+, Rb^+, and Cs^+ in μeq/g Fresh Weight after Different Initial Additions of Phosphate*

One meq/liter of the ion investigated added as the chloride. Radioactivity 10 μc/liter. Na^+ series with 108.5 meq K^+ per liter at all levels of phosphate. Rb^+ and Cs^+ series with 28.5 meq K^+ and 80 meq Na^+ per liter.

Investigated ion	Time (Min)	Initial phosphate conc in mM: 0	0.005	0.05	0.15	0.5	5
Na^+	30	<0.1	<0.1	0.6	2.9	2.9	2.8
	240	<0.1	<0.1	<0.1	1.1	9.0	10.6
Rb^+	60	1.0	...	1.9	...	...	4.7
	240	2.6	...	3.5	...	...	6.4
Cs^+	60	0.4	...	0.7	...	...	1.4
	240	1.3	...	2.3	...	...	4.7

As for Na^+, the uptake compares well with that of the other ions tested at additions of 0.5 to 5 mM phosphate, but when the initial concentration of phosphate is 0.005 mM or below, there is no measurable uptake of sodium. With intermediate additions of phosphate (0.05–0.15 mM at the start), the uptake measured after 30 minutes is greater than if the experimental period is increased to 240 minutes.

These results led to the discovery of a mechanism for the extrusion of sodium by experiments of the type given in table II. During the first minutes of incubation at intermediate levels of phosphate the uptake of Na^+ dominates. After 30 to 60 minutes the sodium is pumped out again, although the cells are still surrounded by the same radioactive solution.

Table II. *Na^+ Taken Up at Different Times after Incubation in a Standard Experimental Medium with Addition of 1 mM $^{22}NaCl$ and 0.15 mM Phosphate*

Conditions as in table I, Na^+ series.

Time (min)	Na^+ (μeq/g dry wt)
30	4.3
65	2.9
125	1.8
180	1.0
230	0.8

and although the proportions between cells and medium are such that the outside concentration of Na^+ is not appreciably affected.

Influence of Phosphate on the Extrusion Mechanism. A closer study of the mechanism for extrusion of sodium met with the obstacle that the cells with P deficiency do not contain or take up measurable quantities of Na^+. To overcome this difficulty, the cells were first prepared as for an experiment, but then the whole batch was resuspended in a standard experimental medium containing 5 mM K phosphates and 1 mM $^{22}NaCl$, and using K citrate buffer. They were incubated at 25° in light, in a 500 ml Erlenmeyer flask open to the air on the shaking water bath but without extra air bubbling. After 2 hours they were again washed twice and the final cell suspension for use in the experiment proper was prepared.

The radioactive cells were now added to flasks with and without phosphate. All experimental solutions contained 1 mM NaCl, but half of them were free from ^{22}Na whereas the other half were labeled. Samples were taken at regular intervals, and the data from one of the experiments are given in table III. The flasks with inactive sodium show that the loss of the ion is enhanced by phosphate, just as the uptake. Also in the presence of labeled Na^+ externally, a loss of sodium from the cells can be demonstrated when no phosphate is present, but 5 mM phosphate will enhance uptake more than outflow, so that a net uptake occurs.

After the pretreatment given, it may seem surprising that the cells show immediate responses towards the presence or absence of phosphate externally. It takes, however, several days in a medium replenished with phosphate before the cells are back in the normally balanced state (11).

Effect of Anaerobiosis. In order to determine whether the mechanism for sodium extrusion is aerobic or anaerobic, experiments were performed in air and under N_2. The cells were loaded with $^{22}Na^+$ by aerobic treatment with a phosphate containing solution as described in the preceding section. Since anaerobiosis will decrease the uptake of external phosphate by *Chlorella* (21), and the same is true for phosphate-deficient *Scenedesmus* (Kylin, unpublished observations), the experiments proper were better performed in phosphate-free solutions.

Results from such series show, for instance, that cells originally containing 21.3 μeq $^{22}Na^+$ per g dry weight retained an amount of 13.1 and 13.5 μeq/g dry weight in air and in nitrogen respectively, after 2 hours in an unlabeled external solution. With 1 mM $^{22}NaCl$ externally, the corresponding figures were 14.6 in air and 15.2 in nitrogen. The extrusion of sodium ions thus occurs both aerobically and anaerobically.

Retention of Rb^+ and Cs^+. Studies on the retention of Rb^+ and Cs^+ were made in order to see whether they can be worked upon by the mechanism for extrusion of sodium. Since these ions are taken up without external phosphate, the cells were made to contain the radioactive ions simply by adding them to the culture media, to a final concentration of 10 μc and 1 meq per liter, about 20 hours before

Table III. *Effect of External Phosphate and Time on Retention and Uptake of ^{22}Na in* Scenedesmus

Cell contents of $^{22}Na^+$ in μeq/g dry weight. Initial loading of the cells with $^{22}Na^+$ as described in text. 108.5 meq K^+ and 1 meq Na^+ per liter at both levels of phosphate.

External sodium	Initial phosphate	Min from start					
		0	30	60	120	180	240
Not radio-	0 mM	19.9	15.2	13.7	11.9	12.0	10.3
active	5 mM	"	12.9	10.0	8.0	6.6	5.4
Radio-	0 mM	"	15.9	15.4	15.1	12.4	12.7
active	5 mM	"	22.2	26.4	30.7	33.4	32.1

Table IV. *Effect of External Phosphate on the Time Dependence of the Retention of Rb^+ in the Presence and Absence of Na^+*

Cell contents of ^{86}Rb in μeq/g dry weight. 1 meq Rb^+ per liter in all series. Na-citrate medium with 28.5 meq K^+ and 80 meq Na^+ per liter; K-citrate medium with 108.5 meq K^+ per liter and no Na^+.

Main buffer	Initial phosphate	Min from start						
		0	30	60	90	120	185	245
Na-	0 mM		5.0	4.8	...	3.7	3.0	2.3
citrate	5 mM	5.7						
			5.0	5.0	...	4.8	4.3	3.6
			4.4	3.4	2.4	1.7	Control with Na-	3.9
K-	0 mM	6.0					citrate at 120 min	4.6
citrate	5 mM		2.7	1.6	1.2	1.0		

the start of the experiment. The cells were then prepared and the experimental solutions made up as described.

Table IV gives some of the data available for rubidium. Using a buffer of sodium citrate, it was found that during a lag of 30 to 60 minutes there was not much difference between the retention in the presence or absence of external phosphate. Thereafter, the phosphate increased the capacity of the cells to retain their Rb^+.

However, when the Na-citrate was replaced by the K-salt, so that the experimental medium became free from sodium, the picture was changed. External phosphate now gave an immediate decrease in the retention of Rb^+ as compared with the phosphate-free solutions. Series like the second one recorded in table IV thus gave a close parallel to the findings for sodium (cf. the nonradioactive set of table III).

The same types of data were found for Cs^+, although the losses were slower, so that somewhat longer experimental times had to be used. For example, an experiment with $(K^+ + Na^+) = (28.5 + 80)$ meq/liter in the medium was started with an initial amount of 5.4 μeq ^{137}Cs per g dry weight. After 130 minutes the cells without phosphate contained 4.1 μeq/g dry weight and those with phosphate 4.7. After 300 minutes the figures were 3.8 and 4.2 respectively. In another set with the medium made up of potassium salts, the corresponding data were 5.3 initially, 3.7 (−P) and 2.9 (+P) at 130 minutes, and 2.5 (−P) and 1.8 (+P) at 300 minutes.

These findings suggest that the same mechanism that is active in the extrusion of sodium can work also upon other monovalent cations, although there is a preference for sodium. When phosphate activates the mechanism and internal sodium is present, rubidium and caesium are excluded from the sites of extrusion, and the cells will retain them more effectively. That the presence or absence of sodium reverses the effect of phosphate on the retention of Rb^+ and Cs^+ would be even more understandable if the latter ions (and, by inference, K^+) are taken up as counter ions to help maintaining the electroneutrality of the cells when Na^+ is extruded.

Effects of Different Combinations of Na^+ and K^+ on Uptake and Retention of Rb^+. It is well known that Rb^+ and K^+ compete with each other for uptake by plant cells (4). In the present experiments there is always an abundance of potassium present, and ^{86}Rb must be regarded as a marker not only for Rb^+ but also for K^+.

With this starting point, some experiments with different combinations of Na^+ and K^+ were made to pinpoint more exactly to which of these ions the results on the retention of Rb^+ (and Cs^+) are primarily due. It could otherwise be argued that the decreased retention of Rb^+ in the absence of sodium, was due to increased possibilities of exchange with external K^+. In regard to the hypothesis that the other alkali ions may serve as counter ions in the active extrusion of sodium, the same combinations were used also for series on the uptake of Rb^+. The result of such studies are given in table V.

Table V. *Effects of Different Combinations of Na^+ and K^+ in the Medium on Uptake and Retention of Rb^+*

Cell contents of ^{86}Rb in μeq/g dry weight. 1 meq Rb per liter in all series. Starting value in retention series: 4.3 μeq/g dry weight. Experimental times: Uptake 200 minutes, retention 120 minutes.

meq/liter in medium		Uptake		Retention	
K^+	Na^+	0 mM P	5 mM P	0 mM P	5 mM P
28.5	80	(1.7)	(4.7)	2.4	3.2
108.5	80	0.8	2.2	2.6	3.3
108.5	0	1.0	1.8	1.0	0.6
108.5	1	1.0	2.4	1.2	1.2

Taking first the aspect of retention, table V shows that with an external Na^+ concentration of 80 meq/liter, it is possible to increase the K^+ from 28.5 to 108.5 meq/liter without any decrease in the retention of Rb^+ within the cells. This excludes an increased exchange with external potassium as the explanation for the effects noted in table IV. One could still argue that the exchange system was already saturated at a concentration of $(K^+ + Na^+)$ of $(28.5 + 80)$ meq/liter, so that no effect could be obtained by the addition of more potassium, but then there would be no reason why the exclusion of Na^+ and the addition of an equivalent amount of K^+ should give the decrease in the retention of Rb^+, which is recorded for $(K^+ + Na^+) = (108.5 + 0)$ meq/liter. It must, therefore, be the presence or absence of sodium ions, which in combination with phosphate regulates the outflow of Rb^+. This conclusion is corroborated by the fact that an Na^+ addition of only 1 meq/liter gives an increased retention of Rb^+, notably when the presence of phosphate has increased the activity of the mechanism for extrusion of sodium. The interpretations given in the preceding section are confirmed by the present experiments.

As for the uptake of Rb^+, the values for the low concentration of external K^+ in table V are given in brackets. This is due to the fact already mentioned that ^{86}Rb must be regarded as a marker for the combined Rb^+ and K^+. In the absence of detailed data on the competition between these 2 ions, it is then possible to make direct comparison only between sets, where the starting amounts of K^+ and Rb^+ in the labeled compartment are the same. When the cells are radioactive and taken from the same batch, as in the retention studies, the condition mentioned is fulfilled throughout. In the uptake studies the flasks with 108.5 meq K^+ per liter can be directly compared, whereas those with K^+ equal to 28.5 meq/liter must be excepted for the time being.

Starting now from the vessels without Na^+, it can be seen that the addition of 1 meq Na^+ per liter to

Table VI. *Limits of Internal Sodium Contents (μeq/g dry wt) Found in Conditions Comparable to Those of Table V*

External Na^+ (meq/liter)	Initial phosphate 0 mM	5 mM
80	3–5	174–191
1	<0.1	10.6–14.2

the medium will increase the uptake of Rb^+ in the presence of phosphate, that is, when the sodium outpump has been stimulated. This gives strength to the idea that alkali ions of the potassium-rubidium- caesium group are taken up as counter ions when sodium is excreted. With Na^+ added to 80 meq/liter there may, again, be a slight inhibition in the uptake of Rb^+, but the effect has not been completely reproducible, and Na^+ does not appear to be very competitive in this type of transport from the medium inwards.

A few more data can be given to illustrate the properties of the system investigated. Table VI gives a picture of the contents of sodium inside cells comparable to those of table V. The series without phosphate especially show that only minute quantities are needed inside to give an appreciable effect on the retention of Rb^+. If we assume $^{86}Rb^+$ to be a proportional marker for the uptake of K^+, the cells should contain 150 to 200 μeq K^+ per g dry weight. Potassium (225–400 μeq) per g dry weight was reported by Krauss and Thomas (9) in their experiments with *Scenedesmus obliquus*.

Discussion

From the evidence given, it appears that both uptake and extrusion of sodium are stimulated by phosphate. At low initial levels of external phosphate, the cells are kept almost free from sodium. With increasing additions of phosphate, uptake of Na^+ increases more than extrusion, and at 0.05 to 0.15 mM phosphate and higher, appreciable amounts are found inside the cells. There is also a time factor involved, so that at an initial concentration of 0.15 mM phosphate the mechanism of uptake is triggered before that of extrusion.

With the conditions used it is not possible to say whether the mechanism for uptake of sodium ions is active or whether it is mainly due to passive influx as a consequence of an increased number of negative charges inside the cell membranes when phosphate is taken up and starts to act. The extrusion mechanism must reasonably be regarded as active, although the data give no information whether it involves transport through membranes or is brought about by metabolically induced changes in the specificity and types of adsorption sites. At the same time, the extrusion of sodium seems to be independent of O_2 supply.

However, the specificity for sodium in the extrusion system is not complete. In the absence of Na^+, both Rb^+ and Cs^+ (and, by inference, K^+) are extruded, although they compete unfavourably with the sodium ions as far as transport from the inside out is concerned (tables IV, V). On the other hand, at least the phosphate-stimulated excretion of sodium is accompanied by an uptake of Rb^+ (and, again by inference, K^+), and for the sites involved in this uptake, sodium can compete at most to a limited extent (table V, uptake column).

From a variety of animal tissues, adenosine triphosphatases have been isolated which are alleged to function in the active transport of Na^+ across the cell membrane (7, 8, 16, 17). The responses of the present system towards phosphate and anaerobiosis would fit such a model, since uptake of phosphate would increase the possibilities of producing ATP in the P-starved cells and since it would not matter whether the ATP is produced aerobically or anaerobically in conditions where the internally available phosphate is limiting.

It has further been established that these adenosine triphosphatases of animal tissues are activated by alkali ions in 2 sites (17). There is one internal site with a preference for sodium, and another external position where K^+/Rb^+ are the ions preferred. There is an obvious parallelism between these biochemical data and the present demonstration, on the physiological level, of a bidirectional transport mechanism, preferentially taking sodium ions out of and rubidium (potassium) ions into the cells.

The above picture is also related to the biophysical data available. Measurements of cell potentials have generally given results indicating that either a permeability barrier or an active mechanism for the extrusion of sodium is working (2). The mechanism demonstrated here supports the alternative of active extrusion of Na^+. Furthermore, it offers a possibility to explain the switching over from one direction to the other of the transport of K^+ which was indicated by the potential measurements of Etherton (5). At high concentrations, K^+ could compete with Na^+ for the active site on the inside of the cell, whereas the transport of K^+ as a counter ion would dominate at low levels of potassium.

A physiological mechanism like the one demonstrated here, gives a new background to earlier findings on the competition between different alkali ions. It was stated by Epstein and Hagen (4) that K^+, Rb^+, and Cs^+ compete with each other for the same absorption system in barley roots, whereas Na^+ inhibits the uptake of Rb^+ in a noncompetitive manner. The interpretation was questioned by Sutcliffe (19), who pointed out that additions of sodium and lithium would increase the total salt contents of the cells, which could hide some of the competitive effects. An increased uptake of Rb^+ due to additions of Na^+ is also demonstrated here, and perhaps the older observations should be re-evaluated and extended to cover more complicated models than competition for only 1 site. Competitive inhibition of the uptake of

Rb^+ to corn leaf tissue by the whole series of alkali ions was, however, recently reported by Smith and Epstein (18).

The uptake of Rb^+ (and Cs^+) as measured in these investigations follows at least *2* different patterns. There is the uptake as counter ion to Na^+, but the main part of the increase due to additions of phosphate occurs as a general response in the uptake of cations at the same phosphate levels which give a net uptake of sodium. More data on the latter phenomenon will be given in another paper. Two pathways for the uptake of K^+ in yeast were postulated by Foulkes (6). As for anions, similar interpretations have been given for sulfate by the present author (10) and for the halide ions by Boszormenyi, Bange and Cseh (1) as well as by Elzam, Rains and Epstein (3).

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