Ion Absorption and Retention by Chlorella pyrenoidosa. I. Absorption of Potassium [1, 2]

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Most of the plant ion absorption experiments in the past have been conducted with coenocytic algae and higher plants (24). In mature cells of these organisms the vacuole occupies 80 to 90 % of the cell volume. With respect to ionic movements the cytoplasm and vacuole represent 2 distinct physical compartments (2, 16). As a result, the molecular details associated with ion absorption, as well as the actual measured rates, are difficult to interpret. In multicellular organisms the interpretation is further confounded by physical obstruction, trapping and movements from cell to cell.

The use of the unicellular alga *Chlorella pyrenoidosa,* for ion absorption experiments avoids most of these difficulties. Young and old cells of growing cultures are similar in structure as seen under the electron microscope. The cells are 2 to 6 μ in diameter and filled densely with cytoplasm. Large vacuoles are absent and the few microvacuoles occupy approximately 1 % of the cell volume (Park, R. B., personal communication). As a result it may be possible to assume, as a rough approximation, that the inner part of the cell forms 1 compartment. In fact, however, the cytoplasm is heterogeneous and includes organelles some of which possess their own permeability barrier. The absence of a large vacuole in *Chlorella,* nevertheless, reduces the complexity of the system by 1 phase.

Chlorella pyrenoidosa has been used in the past for a large number of nutritional and physiological studies. In most nutritional studies the effect of treatments were estimated by some measurement of net growth (6, 12); only in some cases was the change in internal composition measured (11, 21, 22). Thus, in many studies the actual cause of the observed changes or the lack of response to treatments is difficult to ascertain. A study of the short term accumulation of cations will no doubt aid in the interpretation of the extensive literature available.

Materials and Methods

To obtain cells capable of ion accumulation, a low salt medium was developed which permitted the control and variation of the alkali metal cation concentration without changes in the major constituents of the medium (table I). This was accomplished by the use of magnesium as the major cation and urea as a nitrogen source. The latter minimized pH changes observed in media containing nitrates and ammonia.

The cells were grown under sterile conditions in modified gas washing flasks immersed in a water bath. The temperature of the water bath was maintained between 20 and 21°. The illumination (incandescent) was approximately 1500 ft-c. Growth of the algae under the conditions used was vigorous and the dry weight increased approximately 150-fold in 4 days.

The cells were harvested by centrifugation and washed 3 times with distilled water. The concentrated cell suspension was diluted to the desired density using as a guide an approximate packed volume, determined by centrifugation of the suspension in a calibrated tube at 1500 g for 7 minutes. For most experiments a 1 % suspension (based on the packed volume) of cells was used. This was equivalent to

Table I. *Composition of the Growth Medium*

The pH of the medium after sterilization was 6.1 to 6.3. The pH of the medium on aeration with air enriched to 2 % CO_2 was 5.5 to 5.7.

	mmoles/liter*
Urea	3.0
Mg^{++}	3.3
$PO_4^{\equiv}$	2.2
$SO_4^{=}$	2.0
NH_4^{+}	0.3
NO_3^{-}	0.3
Ca^{++}	0.10
Fe (Versanol)	0.11
K^{+}	0.170
Micronutrients**	0.5 ml

* Corrected for salts present in the inoculum.

** Micronutrient Solution	mmoles/liter
H_3BO_3	46.
$MnCl_2 \cdot 4H_2O$	9.1
$ZnSO_4 \cdot 7H_2O$	0.8
$CuSO_4 \cdot 5H_2O$	0.3
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.015
$CoCl_2 \cdot 6H_2O$	0.17

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8 to 10 mg dry weight per 10 ml. The exact dry weight was determined for each experiment. For this purpose, the cells were dried for 48 hours at 75° in weighing bottles. All results are reported as meq per 100 g dry weight.

The experiments were conducted in test tubes (30 × 200 mm) suspended in a temperature controlled water bath at 22°. The light intensity (incandescent) used was 1600 ft-c. For experiments in the dark, the test tubes were covered with several layers of plastic electrical tape and then placed in a dark box.

The gases used for the experiments were freed from undesirable contaminations. Air was passed through KOH to remove CO_2. High-purity water-pumped N_2 was freed of traces of O_2 by passage through alkaline pyrogallol solution and a mixture of methylene blue, zinc, and ascorbic acid in dilute H_2SO_4.

The experiments were carried out in the following manner. The 1 % suspension of cells in distilled water was placed in the tubes and permitted to equilibrate in the temperature bath for 15 minutes. For experiments with N_2 this period was used to deaerate the suspension. Salts were then added to the suspension and this moment was considered as time zero. At appropriate time intervals, samples of the suspension, usually 10 ml were removed with a pipette. The cells were separated from the suspension medium by rapid filtration through a membrane filter (Schleicher and Schuell, A coarse).

The cells were washed on the filter paper 3 times with H_2O or with salt solutions followed by water as required. Table II shows that the washing procedure was quantitatively satisfactory.

The filter paper and cells were placed in Vycor dishes (Corning Glass Works), acidified with 2 % H_2SO_4 in ethanol, dried in an oven and ashed for 30 minutes at 540°. Potassium, sodium, and rubidium were determined by emission flame photometry; magnesium and calcium by absorption flame photometry (5).

Blanks consisting of dishes and filter paper were carried through the normal ashing procedure. The amounts of K, Na, Rb, and Mg found were insignificant. However, the amount of calcium in the membrane filters was not only high, but varied between individual discs. Since the calcium content of the *Chlorella* cells appeared to be low, this variation prevented any reliable estimate of cellular calcium. In the reported exchange experiment (table III), where up to 20 meq of Ca per 100 g dry weight were adsorbed, the experimental error could be as high as 25 %. The phosphate content of the algae was determined by the molybdenum blue, stanous chloride method as described by Johnson and Ulrich (10).

Table II. *Potassium and Magnesium Content of* Chlorella pyrenoidosa *Rinsed on the Filter Pad with H_2O and $CaCl_2$*

Sample no.	No. of H_2O rinses	No. of $CaCl_2$ rinses	Content* meq/100 g dry wt K	Mg
Initial	3		32.4	42.4
Initial		3	32.4	30.4
S 1*	0		52.8	43.2
S 2	1		48.7	41.8
S 3	2		47.2	41.4
S 4	3		46.6	40.8
S 5	4		46.6	41.4
S 6		1	34.2	29.8
S 7		2	34.8	30.8
S 8		3	33.6	30.3
S 9		4	34.2	29.8

* Samples were placed in 5 meq/liter potassium phosphate pH 6.6 for 2 minutes and then harvested by filtration.

Experimental Results

Figure 1 shows the absorption of K by *Chlorella* as a function of time. In about 1 minute 17 to 20 meq per 100 g dry weight of K were absorbed by the cells. This was followed in light-air by a slower phase; under anaerobic conditions (dark-N_2) no further absorption could be observed. If 1 set of samples was rinsed approximately 30 seconds with 5 meq/liter calcium chloride, the slow phase of the curves for water and calcium chloride washed samples were nearly parallel. This suggests that a constant fraction was removed by the salt washing procedure, presumably the K taken up during the rapid initial phase of absorption. The existence of an exchangeable fraction in *Chlorella* has been reported by Scott (21). The difference between light-air and dark-N_2 absorption represents ion accumulation which is dependent on metabolism.

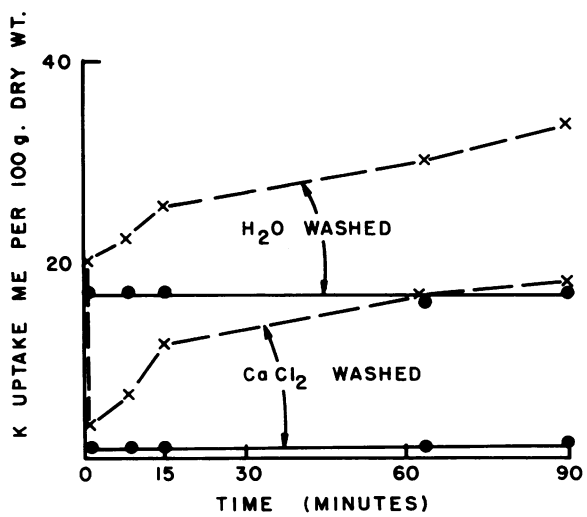

FIG. 1. Uptake of potassium by *Chlorella pyrenoidosa*. Solution composition 5 meq/liter potassium phosphate. Initial K 24.6 meq/100 g dry weight (——•——•— dark-N_2; x---x-- light-air).

Table III. *Quantitative Replacement of Adsorbed Cations*

Sample no.	Order of rinsing solutions*	Content meq/100 g dry wt				
		K	Rb	Na	Mg	Ca**
Initial		28.9	...	...	47.5	13.5
1	Ca	28.9	...	...	32.8	28.0
2	Mg	28.9	...	...	57.2	8.3
3	Mg–K	53.6	...	...	33.6	6.4
4	Mg–K–Mg	28.9	...	...	57.2	10.0
5	Mg–K–Ca	28.9	...	...	32.8	28.0
6	Mg–K–Rb	29.8	23.6	...	33.6	6.4
7	Mg–K–Rb–K	52.4	0	...	32.8	6.4
8	Mg–K–Na	28.9	0	21.3	32.8	5.8
9	Mg–K–Na–K	52.4	0	0	32.8	6.4
10	Mg–K–Na–Rb	29.8	23.0	0	32.8	5.8
11	Mg–K–Rb–Na	28 9	0	20.3	32.8	5.8
12	Mg–Ca	28.9	...	...	33.6	28.0
13	Mg–Ca–Mg	28.9	...	...	57.2	5.8

* Concentration of rinsing solutions 5 meq/liter except for the first K wash which was 25 meq/liter. After final salt washing all samples were rinsed 3 times with H_2O.
** Membrane filter blank not subtracted.

To obtain some insight into the nature of the rapid initial, nonmetabolic absorption, ion exchange experiments were conducted with *Chlorella* cells. Untreated cells suspended in distilled water, were placed with a pipette on the filter pad and then washed with different salt solutions for 30 seconds. Preliminary exchange experiments had revealed that the removal of magnesium from the absorbed fraction was possible by 3 brief rinses with a 1 meq/liter calcium chloride solution. To accomplish the same with potassium chloride required a 25 meq/liter solution. For exchange experiments with monovalent cations, the Mg was first removed from the exchangeable fraction with a 25 meq/liter potassium chloride solution. Table III shows that the adsorbed fraction is of similar magnitude for K, Rb, Na, Ca, and Mg expressed on a meq basis.

Except when stated otherwise, the exchangeable fraction has been removed in all further reported results.

The metabolically dependent K accumulation, in 90 minutes, by *Chlorella* is approximately 16 to 20 meq/100 g dry weight. The size of the metabolically absorbed fraction depends directly on the initial K status of the cells; the higher the internal K content the smaller the metabolic absorption in subsequent experiments (fig 2). If the initial K content is around 47 meq/100 g dry weight the cells will not absorb any additional K (fig 2). Potassium accumulation by *Chlorella* is little affected by the outside concentration between 0.5 and 100 meq/liter (table IV). Although the accumulated K remains constant, the adsorbed K does increase with increasing external K concentration.

Lowering of the pH of the solution from 8.6 to 4.1 decreased K accumulation by approximately 50 % (table V). If Ca was added to the suspension an increase in K accumulation was observed only at pH 4.1. At pH 5.3 to 4.8 Ca had little effect on K accumulation. At still higher pH (6.8) there was about a 10 % decrease in K accumulation (table VI). The accumulation of K from bromide, chloride, sulfate and phosphate salts was the same, provided special precautions were taken to avoid pH changes in the unbuffered solution. During the absorption of K the pH of the outside solution tended to decrease

Table IV. *Effect of Potassium Concentration on K Accumulation by* Chlorella pyrenoidosa
The suspension contained 5 meq/liter potassium phosphate pH 6.8. Initial K was 28.6 meq/100 g dry weight.

Suspension K conc meq/liter	Potassium accumulation meq/100 g dry wt					
	Light-air			Dark-N_2		
	5 min	90 min	120 min	5 min	90 min	120 min
0.1*	7.5	11.7	14.4			
1.0*	5.9	9.8	13.1			
5.0	7.4	14.3	16.2	0.8	0.2	0.8
10.0	7.9	14.3	16.2			
25.0	5.9	16.2	16.2	0.8	0.8	0.8
100.0	7.4	16.2	16.2	3.3	3.3	4.8

* For this treatment the suspension density was decreased to minimize concentration and pH changes.

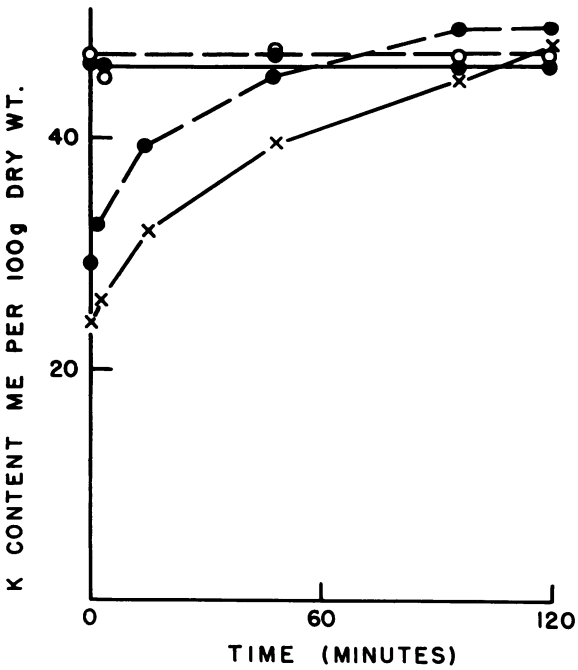

FIG. 2. Effect of initial (equal to zero time) K content on subsequent K accumulation. Cells grown in Meyer's medium (1) containing 20 meq/liter K •——•——•. Cells grown in medium table I containing 1 meq/liter K o---o---o, 0.184 meq/liter K •---•---•, and 0.094 meq/liter K x——x——x. Experimental solution 5 meq/liter potassium phosphate pH 6.8.

in all cases. This suggests that cation accumulation exceeded anion accumulation, which has been confirmed in the case of the phosphate and chloride salts. Under the experimental conditions used *Chlorella* will accumulate, by a process dependent on metabolism, 2.4 mmoles of P per 100 g dry weight in 90 minutes compared with a concurrent K accumulation of 17.5 meq per 100 g. Parallel experiments with Cl showed that not more than 1 meq Cl per 100 g entered the cells as measured with Cl^{36}.

The condition dark-N_2 was used to estimate the effect of metabolic inhibition on ion accumulation by

Table V. *Effect of pH on Potassium Accumulation by* Chlorella pyrenoidosa

The suspension contained 5 meq/liter potassium as phosphate. The initial K was 24.2 meq/100 g dry weight.

pH	Potassium accumulation meq/100 g dry wt		
	1 min	45 min	90 min
4.1	0	7.4	8.6
5.1*	0	10.0	13.3
6.8	0	12.2	17.1
8.3*	0	12.2	18.2

* The suspension density was decreased by a factor of 8 to eliminate pH changes.

Table VI. *Effect of pH and Calcium on K Accumulation by* Chlorella pyrenoidosa

The suspension contained 5 meq/liter potassium as phosphate. The initial K was 28.6 meq/100 g dry weight.

Treatment pH	Potassium accumulation meq/100 g dry wt	
	Minus Ca	Plus Ca*
4.2–4.1	14.5	17.3
5.3–4.8	22.2	22.2
6.8	22.4	20.4

* 1 meq/liter $CaCl_2$ in addition to 5 meq/liter potassium phosphate.

Chlorella. As shown in fig 1 accumulation was completely inhibited. Prolonged exposure to dark-N_2 conditions led to a decrease in the rate of ion accumulation if the cells were returned to air-light (fig 3). The longer the pretreatment in dark-N_2 the slower was the subsequent K accumulation in air-light.

Discussion

Cation absorption (uptake) by *Chlorella pyrenoidosa* has been subdivided into 2 experimentally separable fractions, adsorbed or exchangeable cations and metabolically accumulated cations. The constancy and stability of the accumulated fraction under the conditions used makes the separation of the accumulated and adsorbed ions very satisfactory.

The adsorbed fraction in *Chlorella* has properties very similar to counter ions on polymers. The exchange process is very rapid and is completed in less

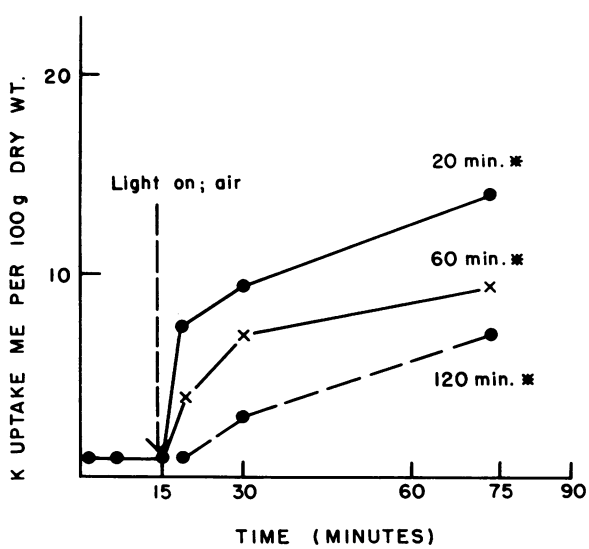

FIG. 3. Effect of pretreatment in dark-N_2-H_2O on subsequent K accumulation by *Chlorella pyrenoidosa*. 5 meq/liter potassium phosphate pH 6.4 added at time zero after dark-N_2-H_2O pretreatment. Initial K 32.6 meq/100 g dry weight. *Time of pretreatment.

than 1 minute. On a meq basis, the size of the adsorbed fraction is similar for K, Rb, Na, Ca, and Mg. The exchange of divalent cations for monovalent cations shows the expected concentration dependence. Changes in metabolic activity have no measurable effect on the size of the adsorbed fraction nor on the rate of exchange. The exact location of the adsorbed or exchangeable ions is not known, however, they are probably found at the surface of the cell. The exchange capacity of *Chlorella* is approximately 20 meq/100 g dry weight (21). The magnitude of the exchangeable fraction falls into the range of values reported for other plant systems (8). However, such comparisons appear of little significance because of the variation of cell or tissue structures and the large differences in the methods of determination (8). The negative exchange sites in large algae and root systems are apparently located in the cell wall (4, 14). It appears, therefore, that if one wishes to compare exchange capacities it might be more appropriate to do so on a cell wall volume or area basis rather than on an entire tissue basis. Such calculations are difficult since the estimation of the cell wall volume is inaccurate and in tissues it is not known whether all cells participate in rapid ion exchange. It is also probable that rapid ion exchange may extend beyond the cell wall depending on the material and the nature of the method used.

Chlorella accumulates 16 to 24 meq/100 g dry weight of potassium in 90 minutes. This entry is dependent on the concurrent function of oxidative metabolism, as shown by the inhibition of net accumulation by dark-N_2 conditions. The accumulated fraction cannot be removed from the cell by rapid washing with salt solutions. Handley and Overstreet (7) have reported that the uptake of Sr, Na, Cl, and Ca in nonvacuolated cells of the meristematic region of the root tip of *Zea mays* is nonmetabolic. However, the uptake they observed appears to be an ion interchange process rather than net accumulation (R. Handley, personal communication). Since the reported metabolic K uptake in *Chlorella* is a net accumulation process, a possible comparison of the 2 systems must await further experimentation.

Exposure of *Chlorella* cells to dark-N_2 decreased the K accumulation when the cells were subsequently placed in air-light. This is analogous to the observation that leaves of higher plants, when kept under dark-N_2 conditions, are damaged first reversibly and then irreversibly in their photosynthetic capacity (15). In general, it appears that in many aerobic cells and tissues anaerobic metabolism is not sufficient to maintain the integrity of the cell.

The metabolic accumulation of 16 to 20 meq/100 g dry weight of K from potassium phosphate was accompanied by an uptake of only 2.4 mmoles of phosphate, or in case of potassium chloride by less than 1 meq of Cl. This small anion accumulation is similar to observations reported for yeast (13, 20). The low Cl accumulation is remarkable because of the low initial Cl content of the cells; 3 meq per 100 g. A possible explanation for this phenomena may be found in a Donnan type co-ion exclusion from the predominantly negatively charged cell content (9). Such exclusion could vary greatly in efficiency depending on the nature of cytoplasmic heterogeneity.

To facilitate the discussion and the understanding of K accumulation by *Chlorella* it appears useful to consider separately the mode of ionic movements, the forces which may induce ionic movements, the means of ion retention, the problem of charge balance, as well as the existence of an upper limit for K accumulation. The transport of ions and molecules can occur by means of diffusion, convection, and propulsion (17). Diffusion requires the existence of an energy gradient such as concentration, electrical or thermal. Accumulation by convection may result from the entry of water into cells and tissues. Convection may also contribute to transport through cytoplasmic streaming (27). A good example of a propulsion mechanism is the suggestion that ions are moved into cells as a result of shape changes of proteins located in the outer membrane (24, 26).

It is suggested that alkali metal cations are accumulated in the cytoplasm of *Chlorella* mainly by diffusion. On the other hand, the movements of ions within the cell may be controlled by convection. The energy gradient necessary for diffusion is created primarily by the synthesis or accumulation of non or slowly diffusible negatively charged molecules in the cell. The cation entry is coupled to the generation of these negative charges. The bonding of cations in the cell would be primarily electrostatic, but coordinate bonds might be of importance, depending on the ion and the type of compound involved. Once an alkali metal cation is in the cell it is retained there as a counter ion and can only be lost if a negative charge is lost from the cell. Alternatively, cations may leave the cell in exchange for other metal cations or possibly hydrogen ions. This concept is supported by the observation that alkali cations are retained by the cell even though the cell is permeable to the ion as measured by isotopic exchange (Schaedle, M., L. Jacobson, in preparation). Therefore, retention in *Chlorella* must be the result of factors other than impermeability of the cell.

As mentioned earlier *Chlorella* accumulates, under the experimental conditions used, more cations than anions. Since electrical neutrality is obviously maintained both inside the cell and in the solution a cation must leave the cell and an anion must be available inside. During net K accumulation in unbuffered solutions the pH of the outside medium decreased continuously. Thus, under the particular conditions K entered and hydrogen ions left the cell (19).

The excess cation uptake in barley (25) yeast (3) and Atriplex (18) is balanced by organic acid synthesis. Presumably, in *Chlorella* negative charges are created within the cell to balance most of the accumulated K. This could occur by synthesis of organic anions or hydrolysis of inorganic polyphosphates or other negatively charged polymers. Protons, created

during the synthetic process or hydrolysis are liberated to the external solution (19).

As shown in figure 2 and table IV K accumulation stops when the cell content reaches 45 to 50 meq per 100 g dry weight. The existence of such an upper limit has been observed in most systems used for ion accumulation studies (23, 24). It appears to be self-evident that ion accumulation in a nongrowing system cannot continue indefinitely. The question of interest, however, is why does accumulation stop at a particular level? To say that it is genetically determined appears trivial, since the nature of the cell in general is determined by heredity. Two observations made during this study may have direct bearing on this problem. Firstly, the upper level of K accumulation in *Chlorella* is relatively insensitive to the outside K concentration. Thus, internal factors must be of predominant importance. Secondly, the cation uptake exceeded greatly the entry of anions thus requiring the generation of anionic sites in the cell. The synthesis of organic anions, or any other reaction sequence capable of creating negative charges in the cell would sooner or later be inhibited probably by the accumulation of the products of the synthetic reaction, either by stimulation of degradative pathways or by a feedback control mechanism. The limited capacity of *Chlorella* to accumulate K could, therefore, be explained on the basis of the limited ability of the cell to create new negatively charged sites possibly organic acid anions.

Summary

Chlorella pyrenoidosa was grown in a low salt medium to obtain cells capable of accumulation. Short-term potassium absorption experiments were conducted to elucidate the nature of the entry process. Net changes in ionic content were determined. For this purpose the cells were separated from the experimental solution by a rapid filtration process.

The following observations were made: Cation uptake by *Chlorella pyrenoidosa* consisted of a rapid initial adsorption phase, followed by a slower accumulation phase. The adsorption process appeared to be similar to cation exchange. It was completed in less than 1 minute, it was not dependent on metabolism and was stochiometric for potassium, sodium, rubidium, calcium, and magnesium on an equivalent basis. The accumulation of potassium, phosphate, and chloride was dependent on concurrent functioning of oxidative metabolism. The rate of accumulation decreased hyperbolically with time and tended to approach zero. The extent of accumulation was determined by the initial content of the cells and not by the concentration of the outside solution. Accumulation of potassium stopped when the cell content reached approximately 47 meq/100 g dry weight. Accumulation of potassium from chloride, bromide, sulfate, and phosphate salts was of similar magnitude.

It was suggested that net cation accumulation was ultimately the result of the creation of slowly or non-diffusible anions, that accumulation was a diffusion process and that retention was the result of the low permeability of the cell to negative counter ions.

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Further Studies Concerning Stomatal Diffusion [1, 2]

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In a previous paper (21), we presented data obtained when water was allowed to evaporate through commercial screens, and interpreted the results in terms of stomatal diffusion. The rule proposed by Brown and Escombe (3) and others (11, 23), that diffusion through single, isolated pores is proportional to the diameter rather than the area was shown to be valid down to 20 μ pores and to extrapolate to the origin at zero pore diameter.

The 5 commercial screens used in this work contained pores from about 20 to 130 μ in diameter, all spaced at approximately 10 diameters, or 200 to 1300 μ on center. The open area was near 1 % in all screens but, because of the expected diameter relationship, the total diffusion through the screens with the smallest pores should have been 7 times that through the screens with the largest pores if Brown and Escombe's (3) conclusion of no interference between pores spaced at 10 diameters was valid. Instead of increased diffusion with smaller pores, we obtained uniform diffusion through all of the screens. This result indicates that the 10-diameter rule is not valid, and that interference increases rapidly when smaller pores are spaced 10 diameters apart. These experiments did not include results with pores more nearly the 5 to 10 μ of average stomates, and yielded no data on the effect of wider spacings.

New screens made to our order, with pores 2.5 to 80 μ in diameter and, for some sizes, 10 to 160 diameters apart, have enabled us to survey the problem of multiperforate diffusion in considerably greater detail than has previously been possible.

Materials and Methods

Electroplated, nickel screens were made by the Buckbee Mears Company of St. Paul. Coated, glass plates were first engraved in squares, then etched, leaving coated squares of the sizes and spacings desired for the pores. These plates were then electroplated to a thickness of 10 μ, forming the primary screens. Some of the primary screens were replated, reducing the size of the pores and increasing the thickness of the screens to a maximum of 80, and an average of 30 to 40 μ. The replating tapered toward the pores (fig 1), and we were unable to measure any effect of the varying thickness. The characteristics of the 23 screens used are shown in table I.

Squares of screen 32 × 32 mm were sealed with a heavy stopcock grease to the tops of special vessels made from 30-mm glass tubing. The vessels contained water at a distance of 10 to 12 mm below the screens. The evaporation and diffusion of water vapor

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