INFLUX AND EFFLUX OF ELECTROLYTES

PART II. LEAKAGE OUT OF CELLS AND TISSUES

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CONTENTS

ABSTRACT

BRIGGS, HOPE and ROBERTSON (1961) consider the uptake of electrolytes to be a combination of active and passive processes, causing simultaneous influx and efflux of salt ions.

In this publication it has been shown, that in *Vallisneria* leaves the peripheral plasma membrane is non-permeable to electrolytes. The continuation of this non-permeability is essential for the efficiency of the active uptake process. The permeability of the membrane is increased by manipulation of the leaves and by changes in the ionic composition of the external solution. A consequence of the non-permeability of the plasma membrane to electrolytes is the active uptake of ion pairs. Ion exchange between plasma and external medium is not involved in active uptake processes of resistant plants. The validity of these principles for active uptake of cations as well as for anions is treated in the discussion.

CHAPTER I

INTRODUCTION

Throughout this investigation on the uptake of salts by *Vallisneria* leaves the view is held that the plasma and cell wall are separated by a membrane. In many objects the plasma membranes can be observed with an electron microscope. In considering the problem of uptake into the cytoplasm it is first necessary to find out what is the permeability of the plasma membrane to electrolytes, and secondly one must know whether passage of the membrane is an active process requiring the use of free energy potentials or a passive process or a combination of both.

When the electrolytes have passed the membrane and are introduced into the cytoplasm, some ions seem to be freely moving in the plasma, others take part in metabolic processes in the cytoplasm. The present investigation of the translocation of electrolytes was confined chiefly to chloride ions. It has been ascertained that after chloride ions have been taken up into the cytoplasm they move rapidly over fairly large distances. (ARISZ, 1960, 1963b).

The capability of the plasma to allow for such rapid movement is presumably based on the structure of the plasma micelles which offer little resistance when electrolytes move through them.

Polarity of translocation has not been found in *Vallisneria* leaves. Movement of chloride ions in the plasma occurs in all directions, just as in a diffusion process, and it depends similarly on a concentration gradient (ARISZ, 1963b). This applies to chloride ions as well as to cations like potassium and rubidium, and dipolar ions such as those of asparagine and aminoacids. Seeing that the specific qualities of the ions and their reactivity are maintained during their movement, it is unlikely that the ions are chemically bound to carriers during their migration.

The cytoplasm of adjoining cells is connected by plasmodesmata and forms a continuous whole which is called the symplasm; the sieve tubes also belong to this symplasmatic system. In former experiments by ARISZ and SCHREUDER (1956) it was found that the translocation of chloride in the bundles and parenchyma cells of *Vallisneria* leaves display no essential differences; for this reason, smaller differences between translocation in sieve tubes and in parenchyma cells will not be discussed here.

In Part I, the permeability of the plasma membrane to electrolytes was discussed with reference to the critical survey of permeation of non-electrolytic organic substances, made by WARTIOVAARA and COLLANDER (1960). These organic substances are absorbed according to their affinity to lipids present in the membrane and in the cytoplasm (HÖFLER, 1960, 1961). Some can penetrate through the pores in the membranes, which are usually small and are only passed through by small molecules such as those of water, which possess a low affinity to lipids. It may be that the pores are not fixed structures in the

membranes but appear and disappear continuously (Wartiovaara and Collander).

HÖFLER has pointed out that we have to discern a lipid pathway and a pore pathway. Since lipid substances can be found everywhere in the protoplasm, it is evident that lipophilic substances can penetrate to all parts of the protoplast.

The introduction of electrolytes into the cytoplasm offers difficulties because of their low affinity to lipids and because of their dissociation into ions which cannot pass through the pores on account of their electric charge.

The presence of lipid membranes surrounding the organelles prevents the distribution of electrolytes throughout the whole of the protoplast. The possibility that electrolytes play a more specific role in cell metabolism than organic lipid soluble substances, which can penetrate everywhere is suggested by the inner structure of the protoplasm which has a great number of separate "compartments". Such protoplasmic compartments are nucleus, vacuoles, mitochondria, plastids, granules and endoplasmatic reticulum, which are probably all bordered by specific membranes. The fairly high impermeability of lipid membranes to non-lipid-soluble substances, stressed by COLLANDER, reveals the possibility that electrolytes may accumulate in some of these "compartments". Electrolytes would then need an energy consuming mechanism to pass the plasma membrane. An often quoted hypothesis regarding the uptake of electrolytes supposes that ions pass a membrane by combining with lipid carriers. In several publications on uptake, the presence of a carrier system is accepted, but the part played by the membrane (plasmolemma or tonoplast) is not mentioned, and it is not stated whether the ions, once they have penetrated into the plasm continue to be bound in ion-carrier complexes or are released immediately after the passage. If the ions remained combined with the carriers, they would continue their journey into all the cytoplasmic compartments of the cell, thanks to the lipid affinity of the carriers. This seems unlikely, and is not in agreement with the metabolic regulation of the localisation of electrolytes in the different compartments.

Also, the results of the long distance transport of chloride ions in the symplasm without any loss of ions to the external solution do not fit in with the hypothesis of a stable combination of ions with carriers in the cytoplasm. Application of the carrier hypothesis has also to be considered for the passage of the tonoplast.

According to the multi-step theory (ARISZ, 1953, 1956, 1958, 1960, 1963) the ions, after having been absorbed into the cytoplasm, may have to pass other membranes e.g. the tonoplast. The carrier theory would then require the formation of a new ion-carrier complex, functioning only during the passage of the vacuole membrane. Once inside the vacuoles, the chloride ions with their partners are free, and it is evident that a simultaneous displacement of cations and anions is always necessary, (cf. Discussion D).

An efficient metabolism requires membranes impermeable to

electrolytes. This means that the cytoplasm with its compartments cannot be a free space for electrolytes. It is highly interesting that MAC DONALD and LATIES (1963), investigating the uptake of chloride ions into potato slices found that there was a period of rapid uptake (the absorption shoulder) followed by a period of steady state uptake; they conclude that both these phases of anion absorption are under metabolic control.

BRIGGS, HOPE and ROBERTSON (1961) have given the general view that in the cell active and passive uptake processes accompany each other. If only the direction of these processes is under consideration, and their connection with metabolism and the question of whether they cause uptake or loss from the cells, is ignored, they speak of influx and efflux. They say, "the accurate knowledge of influx and efflux is necessary for understanding what is happening in a cell during uptake. The net flux is dependent upon a number of con-ditions, influencing the rate of influx by active transport and causing efflux by rising the salt concentration in the cytoplasm and by changing the resistance to passive efflux".

Influx and efflux must be estimated for each kind of ion separately and at the same time one must consider whether the fluxes are active or passive.

In part I it has been proved that during uptake of chloride ions in *Vallisneria* leaves the outer plasma membrane is not permeable to chloride ions. There is no measurable efflux of chloride ions during that time. This means that the uptake of labelled ions into the plasma cannot be accomplished by exchange of unlabelled ions in the plasm with labelled ions in the external solution. Further, it has to be considered whether the same rule is valid for other ions, and whether it is a general rule for all kinds of ions that passing through intact plasma membranes is an active process, using free energy potentials.

The aim of this publication will be to show that in *Vallisneria* leaves the state of impermeability of the plasma membrane can be changed temporarily. The various causes which bring this about are a renewal of the external solution (ARISZ, 1943), or a change in its composition, or a change in the relation of mono- and divalent cations in it, or other more direct influences such as manipulation or cutting of the tissue, irritation by an electric current or sudden changes of tempera-ture. After a short time the original condition may be restored again (STILES).

In 1943 a change of activity in passive uptake processes was studied with regard to the uptake of asparagine, while the material used for the experiments was insufficiently resistant. A passive passage of the peripheral membranes in both directions was found and ascribed to increased permeability of the plasma membrane.

From that time we have, as far as possible, avoided working with insufficiently resistant *Vallisneria* material, since we were convinced that the use of tissue with increased permeability could adversely affect the reliability of the results. Though we have not succeeded in obtaining resistant material throughout the whole year, there have

been long periods in which all the material available was of high quality and could be used in experiments lasting several days without reacting to changes in the external solution by increasing in permeability. The material was, in general, least resistant during the winter between November and March. In some years, highly resistant material could not be obtained on account of either infection by viruses or epiphytic *Algae*,[1]) or because of abnormal soil conditions.

In discussing the results of the experiments on leakage we shall come to the question of whether the variable results caused by leakage are an isolated phenomenon confined to *Vallisneria* leaves, or are a common feature of uptake experiments in different kinds of plants. This last suggestion seems to be the case, since analogous results have been obtained from work carried out on isolated cells of members of the *Characeae*, and on cells from roots, leaves and storage tissues.

In working with all these materials the same care ought to be used as in experiments with *Vallisneria* leaves, to distinguish results obtained with resistant material from those obtained with material of which the plasma permeability has been changed.

Although statistically reliable results can be obtained by using large numbers of variants, there is no guarantee that the use of large quantities of plant material will give a deeper insight into physiological processes. For example, when the material is not homogeneous in respect of membrane permeability, no reliable data about the process of active uptake can be obtained without a thorough physiological investigation of material which *is* homogeneous in this respect. It was this line of thought which led to the following experiments.

<div align="center">CHAPTER II</div>

METHODS

The methods used have been described in preceding publications (ARISZ, 1947, 1953, 1958). Pictures of the uptake- and pre-treatment vessels have been published in the first part of this research on influx and efflux (ARISZ, 1963a), and here we shall confine ourselves to a short survey.

After cutting the leaves into segments of uniform length (2.5 cm) and breadth (0.4 cm), the segments were then randomized in the usual way (ARISZ and OUDMAN, 1937) according both to the leaves from which they were cut and to the distance from the tip of each leaf.

Eight leaf segments of 2.5 cm length form a series, which is mounted in a perspex frame. These are handled as a unit throughout the experiment.

After cutting the leaf segments and composing the series, the frames

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[1]) I have to thank Miss Dr. J. TH. KOSTER, Leiden, for the kindness of identifying these algae as *Oedogonium* sp.

were, in most experiments, placed in a horizontal position in a large perspex trough, 30 cm long, 16.3 cm wide and 3.9 cm high, for pre-treatment. The trough was placed on an opal glass plate on a support and a T.L.F. tube (Philips 40 W/33) mounted under the plate. All the leaf segments were thus illuminated uniformly on their underside; the increase in temperature was very slight.

For the uptake of chloride ions, labelled with Cl 36, square perspex boxes of 8 cm length and breadth and 2.2 cm height were used. They contained about 100 ml of solution and two frames of leaf segments, placed horizontally a small distance from the bottom. During the uptake period the solution was aerated and stirred by introducing a flow of air bubbles from a vertical tube; this caused a circulation of the solution through the vertical and horizontal tubes and the vessel.

The perspex vessels with their two leaf series, were placed side by side on the opal glass plate and illuminated from below by a T.L.F. tube. After the uptake experiment the leaf series were transferred into water or salt solutions for rinsing and exchanging. The leaf segments were then transferred to glass tubes and each series was digested at a temperature of 92° C in 2 ml 0.05 N AgNO$_3$ containing nitric acid, for a period of 2$\frac{1}{2}$ hours. The amount of chloride was estimated by the Volhard method.

The absolute values obtained are subject to a systematic error; however, when the increase of chloride ions in the tissue is being determined the method is more reliable as the difference of two estimations is used, thus removing the effect of a systematic error.

After the Volhard estimation the precipitate was dissolved in ammonia and the radioactivity of the solution was measured by means of a dipping Geiger-Müller tube. The chloride content of the solution used for the uptake was estimated by the Mohr method. By measuring the activity in counts per minute the amount of labelled chloride ions in μg per series of leaf segments could be calculated.

CHAPTER III

MEASURING THE LEAKAGE OF CHLORIDE DURING UPTAKE OF LABELLED CHLORIDE IONS, BY COMPARING THE FLUX OF LABELLED CHLORIDE IONS WITH THE FLUX OF TOTAL CHLORIDE IONS

In the first part of this investigations (ARISZ, 1963a) the following method was developed to assess the permeability of the plasma membrane to chloride ions during chloride uptake by leaves of *Vallisneria spiralis.*

The principle of the method consists of putting the tissue into a chloride solution labelled with chloride 36 and comparing the amount of labelled ions absorbed (C) with the increase of chloride ions in the cell, which may be estimated by chemical methods (B–A). In

other words the flux of labelled chloride ions is compared with the net flux of total chloride ions. The amount of labelled chloride ions absorbed is determined by measuring the radioactivity of the tissue after the uptake.

From the radioactivity measurements the amount of labelled chloride absorbed can be calculated by means of the specific activity of the external solution.

The increase in the amount of total chloride ions in the cell is chemically estimated by the Volhard method; it is in fact, the difference between the amount of chemically estimated ions in the tissue at the start of the uptake (A), and that at the end of the uptake period (B). The increase of total chloride during uptake is B–A and represents the net flux of total chloride ions during the uptake period, which is the result of the influx, and a possible efflux of chloride ions.

It will be shown that with good material at the beginning of an experiment, the same amount of unlabelled chloride ions (A) is present in all the series of leaf segments involved in the experiment. If the chloride ions in the tissue are enclosed within a plasma compartment and thus separated from the external solution by a membrane impermeable to chloride ions, there will be no leakage of these ions from the tissue. But as soon as the peripheral plasma membrane becomes more or less permeable to chloride ions, a loss of chloride ions will result. Unlabelled chloride ions, already present before uptake, and labelled ions just absorbed, will leave the tissue together with an equal amount of cations. The more the membranes of the compartments are permeable to chloride ions, the more the amount of chemically estimated chloride ions will decrease. A loss of labelled chloride ions by exchange will be discussed later.

If, at the end of uptake, the increase in the amount of chemically estimated (viz. total) chloride ions in the tissue is equal to the amount of labelled ions absorbed, (B–A) = C, it may be concluded that during the uptake period no loss of chloride ions has occurred; in other words, if the flux of labelled chloride ions equals the net flux of total chloride ions the membrane is impermeable to chloride ions. The net flux of total chloride ions is the result of the influx of chloride ions which is offset by an efflux of chloride ions. If the net flux of both of total ions and of labelled ions are equal, there is no efflux, which means there is no leakage of chloride ions.

Since the absorbed labelled ions will be distributed over cytoplasm and vacuole, where a considerable quantity of unlabelled chloride ions were already present before absorption began, the behaviour of these unlabelled chloride ions in the tissue is used as indicator of the movement of the labelled chloride ions. If unlabelled chloride ions leak from the cells, it has to be expected that the newly absorbed labelled chloride ions will do the same.

A loss of labelled ions from the tissue during uptake of labelled ions cannot be detected by this method since the quantity of chemically estimated chloride ions will decrease by the same amount as that of labelled ions. It is unlikely that labelled ions will leak from the plasm

when there is no simultaneous loss of unlabelled ions, since the plant cannot distinguish between labelled and unlabelled ions.

If the increase of chemically estimated chloride ions (flux of total ions) is smaller than the uptake (flux) of labelled ions, (B–A) < C, a loss (efflux) of unlabelled chloride ions, which were present in the tissue before the uptake, must have occurred. This is an indication that labelled chloride ions may also have left the tissue during the uptake period.

Table I from ARISZ (1963) (I) is reproduced here. It contains some data about five experiments on chloride uptake, which differed in the duration of pre-treatment (7 to 24 hours) and in the composition of the external solution used. This contained potassium, calcium and sulphate ions, or consisted of a solution of calcium nitrate. The absorption period varied from $11\frac{1}{2}$ to 22 hours, while the concentration of the labelled potassium chloride solution was about 1.5 mM in all experiments. The temperature was 25° C.

In all experiments the uptake of labelled chloride equalled the increase of the amount of chemically estimated ions. This means that during the uptake no change in permeability of the membrane has occurred. The membranes are practically impermeable to chloride ions, indicating that during uptake no measurable efflux has occurred.

If part of the uptake of labelled chloride ions was the result of an exchange of chloride ions in the tissue for labelled chloride ions from the external solution, the total amount of (chemically estimated) chloride ions would not have been altered by that exchange.

Since the labelled ions will increase if an exchange takes place, C would then become larger than B–A. *The fact that B–A and C in Table I are equal proves that in the uptake period no chloride exchange has occurred.*

The same result was obtained in an experiment with 16 series of leaf segments. (Table II). After cutting the leaves and composing the series, these were pre-treated for twenty four hours in the light in 1 mM calcium nitrate solution. Two series were used for estimating, by the Volhard method, the quantity of chloride present per series after 24 hours pre-treatment.

After pre-treatment, the remaining 14 series were immersed in a solution of potassium chloride and calcium sulphate containing 1.6 meq. Cl labelled with chloride 36. In each uptake vessel the two series were illuminated from beneath while the solution was continuously aerated and mixed (cf. Fig. 1 ARISZ, 1963a). The results are given in Table II.

Comparing the results of the different series, it is apparent that the first 12 series agree satisfactorily and show an equal value both for the increase of the total chloride ions and for the absorbed labelled chloride ions (285 ± 1.46 μg chloride per series). The two series 8a and b at the foot of the column show a significant difference; 270 and 279 μg labelled chloride per series was present, and the flux of total chloride amounted to 256 and 248 μg respectively.

Since the 12 first mentioned series did not leak during uptake as

TABLE I

Impermeability of the peripheral plasma membrane to chloride ions during uptake of labelled chloride.

The flux of labelled chloride ions (C) is equal to the flux of total chloride ions (increase of chemically estimated chloride ions B–A), indicating that no efflux of non-labelled chloride ions occurs during uptake of labelled chloride. Pre-treatment in Exp. 90, 91 and 92 in potassium sulphate with calcium sulphate, and in Exp. 93 and 94 in calcium nitrate for periods of 7 to 24 hours.

Uptake in different concentrations of potassium chloride labelled with Cl 36, and the addition of calcium sulphate during periods of 7 to 24 hours. (From ARISZ, 1963, Protoplasma 57).

Exp. Nr	pre-treatment period		concentration labelled KCl solution	absorption period hours	Volhard estimations μg Cl /series			labelled Cl absorption μg Cl /series	after uptake rinsed in
	hrs.	solution			A before absorption	B after absorption	B–A increase Flux total Cl	C Flux labelled Cl	
90	24	$K_2SO_4 + CaSO_4$	1.65 mM	16	451	760	309	312	$K_2SO_4 + CaSO_4$
91	10	$K_2SO_4 + CaSO_4$	1.43 mM	11½	376	467	91	92	$K_2SO_4 + CaSO_4$
92	16	$K_2SO_4 + CaSO_4$	1.74 mM	22	355	645	290	291	$K_2SO_4 + CaSO_4$
93	7	$Ca(NO_3)_2$	1.62 mM	20	387	572	185	185	$Ca(NO_3)_2$
94	24	$Ca(NO_3)_2$	1.57 mM	21	309	612	303	303	$Ca(NO_3)_2$

TABLE II

Variability of chloride uptake.

After cutting the leaves to standard dimensions the 16 series have been pre-treated in 1 mM calcium nitrate solution in the light for 24 hours. During a period of 21 hours in the light fourteen series have absorbed chloride from 1.6 mM potassium chloride solution, labelled with Cl 36 and containing calcium sulphate. After uptake the series have been rinsed in water for two minutes; each uptake vessel contains two series a and b. In series 8a and b leakage occurs, since B–A < C. Exp. W 95.

			μg Cl /series (Volhard det.)			μg labelled Cl /series	
			A	avg			
1. Chloride content after pre-treatment		a b	351 344	348	Flux total Cl	Flux labelled Cl	
			B		B–A avg	C avg	
2. Chloride content after uptake and 2 min. rinsing		a b	636 636		288 288 288	288 290 292	
3.	idem	a b	636 628		288 284 280	281 281 280	
4.	idem	a b	632 621		284 279 273	285 283 280	
5.	idem	a b	636 632		288 286 284	292 291 291	
6.	idem	a b	643 639		281 286 291	283 281 279	
7.	idem	a b	639 636		291 290 288	281 285 289	
8.	idem	a b	604 596		256 252 248	270 275 279	

(B–A) = C, and at the end of the uptake period contain an average of 285 μg labelled chloride, it is likely that the last two sets in which (B–A) < C did, in fact, leak.

By comparing the amount of chloride ions in the leakage series with that in the series which did not leak the amount of leakage can be assessed. It amounts to 15 resp. 6 μg (avg 11 μg) labelled chloride and 37 resp. 29 μg (avg 33 μg) chemically estimated chloride.

Reconsidering the first 12 series, one gets the impression that in series 4b also a small loss of ions may have occurred. It seems likely that during uptake, the permeability of the plasma membrane was not altered, but that in some sets during the time between uptake and the destruction of the material, i.e. while the tissue was rinsed, a slight change in permeability of the plasma membrane occurred.

These results are instructive since they give an indication of the degree of accuracy of the determinations, and because it is apparent that a slight alteration suffices to change a non-leaking series into a leaking one.

The series 8a and b show a loss of labelled chloride ions with a lower specific activity than the specific activity of the labelled external solution. This must be the result of unlabelled ions being present

before uptake began in the plasma compartment into which the labelled ions have been introduced.

It may be expected that there is a continuous secretion of chloride ions from the plasmatic compartment into the vacuole. This is proved by the increase in the osmotic value after salt uptake. The spec. act. of the solution in the vacuole will therefore be lower than that in the plasma compartment.

Leakage may of course, occur through both membranes, but it may be expected that in this experiment the slight leakage is mostly caused by leakage from the plasma compartment.

The question of how large the pools of ions in the plasmacompartment and the vacuole are, and how the specific activities of these solutions are changed in the course of the uptake process, cannot be answered here.

<center>CHAPTER IV</center>

SECONDARY CHANGES IN PERMEABILITY AS A RESULT OF CELL INJURY

The increase of the chemically estimated chloride during uptake is worked out from the amounts of chemically estimated chloride present at the start and finish of the uptake period. The reliability of these determinations depends on the correctness of the assumption that in all series of an experiment an equal amount of chloride ions is present at the start of uptake. This point will be discussed here. The series were composed of eight leaf segments of 2.5 cm length and 4 mm breadth. They were cut from 8 leaves and were situated at different distances from the top of the leaves. With an apparatus composed of two parallel Gillette blades fixed at 4 mm distance from each other the leaves are cut over the whole length to a breadth of 4 mm and then divided into segments of 2.5 cm length.

The wounds inflicted by this treatment have a great effect on the power of the leaf segments to absorb chlorides actively. Some cells are heavily injured and lose their contents, other cells are only slightly injured and display exosmosis from the symplasm.

During the pre-treatment of the material in water or other solutions a recovery of these cells follows and the impermeability of the plasma membrane is restored. Since the losses of cell contents in leaf segments of resistant material are almost equal, the amount of chemically estimated chloride per series will also be more or less equal after 24 hours pre-treatment. Only when the material is less resistant does an exosmosis of variable duration and degree result, and this makes the amounts of chloride ions per series also rather variable. In one experiment (W 62) 10 series were compared, and they contained 624.9 $\pm$ 3.2 μg chloride.

In ordinary experiments only 2 series were tested in order to know the amount of chemically estimated chloride ions present at the start

of the experiment. For leaves in good condition this is sufficient, but
when the material is not resistant, exosmosis ensues in periods of
different length, causing a variable amount of chloride in different
series of the same experiment.

The duration of the wound influence can be investigated by deter-
mining the amount of chloride ions in the leaf series after various
times of rinsing. Table III contains data of two experiments showing

TABLE III

Loss of chloride which resulted from cutting the leaves and composing the series,
    and which was measured during rinsing periods of different lengths.
  The series have been rinsed in 1 mM calcium nitrate solution. Two experiments:
W 108 and W 105.

duration of rinsing period after cutting	μg Cl /series exp. W 108	μg Cl /series exp. W 105
fast rinsing	277	203
5 min. rinsing	244	
1 hour rinsing	231	193
4 hours rinsing		194
24 hours rinsing	243	

a wound effect, which consisted of the loss of the cell contents from
the cut cells, and of exosmosis from the adjoining cells. After an hour
the leakage has diminished, and a reabsorption has appeared if
chloride ions are present in the external solution. This is promoted
if the leaf series are placed in a small volume of solution after cutting.

According to our standard treatment, after the series have been
cut, they stay in water during a pre-treatment period, and generally
several series are pre-treated in the same trough. Some less resistant
leaf segments will lose a considerable part of their chloride content,
while other segments remain active and are able to accumulate the
chloride ions lost. Rather large differences in the chloride ion content
of the series will then arise. If the material is resistant and in fairly
good condition, no difficulties result from placing a large number of
series in the same trough, but when good and leaking material are
mixed, the results will not be reliable. COLLANDER (1939) had the
same experience with cells of the *Characeae*. This means that during
all manipulations, such as rinsing and exchanging, the mixing of
resistant and leaking material will have to be avoided, since it has
an unfavourable influence on the accuracy of the results.

It may appear possible to prevent these difficulties from arising by
a timely renewal of the external solution, but it must be realized that
this renewal may also involve other effects. It will be shown that
leakage may be caused by loss of calcium ions from the tissue. This
loss of calcium ions decreases after some time, probably in relation
to the increasing calcium content of the outer solution. In renewing
this solution the calcium balance is again upset and calcium leakage
will be resumed, as the permeability of the plasma membrane increases.
To prevent loss of calcium ions from the plasma membrane calcium

nitrate or sulphate can be added to the external solution. In *Vallisneria* leaves the anions of these salts are only accumulated in small quantities.

A second point is in what way the capacity for active uptake is influenced by cutting and rinsing the leaves. The effect of the length of a pre-treatment period in water has been previously investigated (ARISZ, 1947). Table IV gives the result of an experiment with pre-

TABLE IV

Recovery of active uptake after cutting the leaves.
Influence of the duration of pre-treatment in water on uptake of chloride from 1 mM potassium chloride solution (with addition of calcium sulphate) during a period of 24 hours. (From ARISZ, 1947. Table 2. Exp. M 43).

duration of pre-treatment	chloride uptake in μg Cl /series
not in water pre-treated	98
2 hours in water pre-treated	120
4 hours in water pre-treated	131
7 hours in water pre-treated	142
24 hours in water pre-treated	246

treatments of 0, 2, 4, 7 and 24 hours in length followed by an absorption for 24 hours from a 1 mM KCl + CaSO$_4$ solution; the net flux of the chloride absorption increases with the length of the pre-treatment. It is not clear whether the increase of the net flux is the result of diminishing efflux or of a recovery of the active uptake process.

In Table V the influence of one pre-treatment of 5 one of 20 hours duration have been compared. The pre-treatment was followed by uptake during a period of 24 hours in the light from a 1.4 mM KCl + CaSO$_4$ solution labelled with Cl 36.

If leakage occurred during uptake the increase of the chemically estimated chloride should have been smaller than the uptake of the labelled chloride ions, but this did not happen either after 5 or after 20 hours pre-treatment, since in both cases the increase of the chemically estimated chloride ions equalled the uptake of labelled chloride ions. After 5 hours pre-treatment 200 μg Cl* and 201 μg Cl have been found and after 20 hours pre-treatment, 251 μg Cl* and 256 μg Cl were found. This means that leakage had stopped completely, in this case, in less than 5 hours, but that the rate of the active uptake process was higher after 20 hours than after 5 hours pre-treatment.

Besides the recovery of the plasma membrane, restoration of the free energy potentials necessary for the uptake processes is also needed.

The resistance of the leaf material used and the composition of the external solution during the pre-treatment have an influence on the length of the recovery period.

In experiment W 112 Cl uptake was found to be equal after one, four or 22 hours pre-treatment in calcium nitrate. No leakage occurred since, in this experiment, the uptake of labelled chloride was in all three cases as large as the increase of chemically estimated chloride.

TABLE V

Recovery of the power for active uptake during pre-treatment in calcium nitrate.
    I.   Pre-treatment 20 hours in the light in 1 mM calcium nitrate.
    II. The same pre-treatment for a period of 5 hours.
The pre-treatment is followed by 24 hours' uptake in the light from 1.4 mM KCl, containing labelled chloride and calcium sulphate.
After uptake the leaf series is rinsed for two minutes in the light in calcium nitrate.
The equal values of the flux of total chloride and the flux of labelled chloride proves that leakage has not occurred during uptake, and that the higher uptake after longer pre-treatment is a consequence of the recovery of the power of active uptake. Exp. W 101.

| | | Volhard estimations | | Flux total Cl B–A µg Cl/series | Flux labelled Cl C µg Cl/series |
		A µg Cl/series before absorption	B after absorption		
Cl content/series after	a	288 286			
pre-treatment	b	284			
I. Uptake after 20 hours	a		540 542	256	248 251
pre-treatment . . .	b		543		253
II. Uptake after 5 hours	a		490 487	201	202 200
pre-treatment . . .	b		483		198

In this case even after only one hour pre-treatment the loss of chloride was stopped and the power for active uptake had returned completely.

It must be stressed that the recovery of the structure of the plasma membrane which restores its primary impermeability to chloride ions, is not identical with the restoration of the power for active uptake. In dealing with the translocation of chloride ions it has been shown that the power of translocating ions in the symplasm depends on still other factors and needs a longer time of recovery (cf. Fig. 1 from ARISZ, 1947).
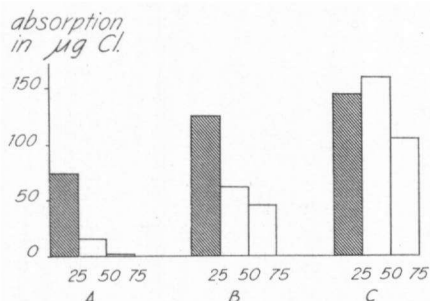
Fig. 1. Recovery of uptake and movement in the symplasm after cutting the leaves to standard dimensions. After cutting the material into leaf segments of 7.5 cm length and 4 mm width uptake starts at once in A, in B after 16 hours pre-treatment in water and in C after 24 hours in water. Uptake by the absorbing zone from a 10 mM solution of potassium chloride with calcium sulphate, and translocation of the absorbed chloride in the adjoining free part of the leaf. The uptake zone is in the dark, the free part is exposed to light. (From ARISZ, 1947, Proceedings Kon. Ned. Akademie, Vol. 50, fig. 2.)

CHAPTER V

INTACT AND CUT LEAVES

Since most experiments have been performed with leaf segments of a standard length and breadth, it may be asked whether the cutting and wounding have had any influence on the uptake process. It can be established beyond doubt that uptake is not a consequence of the wounding, and that intact leaves give an analogous result.

It has been shown by means of autoradiographs (ARISZ, 1961a, b, 1963b) that in an intact plant, labelled chloride and labelled rubidium ions can be locally absorbed by a part of the leaf and are translocated in the symplasm of the leaf cells for considerable distances (25–60 cm in 24 hours) to the younger leaves and through stolons to the growing young plants attached to them.

As the plants were placed in a basin of water, thus preventing transpiration, and as, according to SOLEREDER, the xylemvessels of these waterplants are reduced, the translocation could not be the consequence of suction forces, and its path was not along the walls or the xylem, but in the symplasm. The rate of translocation measured over a period of 24 hours was about 2.5 cm/hour, which is more rapid than the rate of diffusion within a solution.

No radioactive chloride ions once absorbed were returned to the external solution during translocation, indicating that leakage through cell walls and passive permeation through the plasma membranes were out of the question. The translocation process will be considered more fully in a following publication. Here, we confine ourselves to mentioning the possibility of a strong active uptake and transport in intact tissues, not caused by cutting and injuring the tissue and occurring in tissue which has not undergone pre-treatment in water. Active uptake and symplasmatic transport are fundamental properties of the protoplasm (cf. ARISZ, 1963b).

Identical results had already been obtained over shorter distances in quantitative experiments; they were performed with series of leaf segments cut to a standard size. Large wounds are inflicted by cutting the leaves to obtain a uniform width of 4 mm and a standard length of 2.5 cm over their whole length. It is worth while tracing how these wounds influence both the amount of chloride ions present in the leaf at the beginning of the uptake, and also the power of the cytoplasm to introduce chloride ions from the external solution through the plasma membrane into the symplasm. As the material stays in water or calcium sulphate or calcium nitrate for a rather long time (generally 24 hours) after cutting, the wound influence will have disappeared before the uptake begins. It can be expected that series of randomized leaf segments, which have the same leaf area, will display about the same uptake capacity during an experiment, depending on conditions of light, temperature, osmotic concentrations, free energy potentials and so on, and which may differ for intact and cut leaves.

TABLE VI

Influence on chloride uptake of cutting leaf segments to a uniform width of 4 mm.

I. All leaf segments retain their natural width, which is rather variable. In 1, 2, 3, 4 there are 4 segments of 5 cm length, which, after uptake, are cut into two parts of 2.5 cm. A series is formed by 4 segments of 2.5 cm length which retain their natural width.

II. Leaf segments cut at uniform width of 4 mm before the pre-treatment. Series 5, 6, 7, 8 consist of 8 leaf segments of standard length (2.5 cm) and standard width (4 mm).

During pre-treatment all series are rinsed in 2 mM calcium nitrate for 24 hours. Uptake in series 2, 3, 4, 6, 7, 8 took place from 2.365 mM KCl solution, labelled with Cl 36 and containing calcium sulphate. After uptake, the series were rinsed in 1 mM calcium nitrate for 5 minutes, 1 hour or 20 hours. Exp. W 84.

		Volhard estimations μg Cl/series		Flux total Cl	μg $\dfrac{\text{Cl/series}}{\text{Flux}}$ labelled Cl	mg fresh weight
		before uptake A avg	after uptake B avg	B-A	C avg	
I. Original breadth						
1. Cl content/series after	a	249 248				200
pre-treatment . . .	b	247				
2. after uptake 5 min.	a		639 643	395	a 387 392	218
rinsed	b		646		b 396	
3. after uptake 1 hour	a		593 590	342	a 340 338	208
rinsed	b		586		b 336	
4. after uptake 20 hours	a		572 579	331	a 331 340	206
rinsed	b		586		b 350	
II. 4 mm breadth						
5. Cl content/series . .	a	376 375				260
	b	374				
6. after uptake 5 min.	a		731 731	356	a 364 358	259
rinsed	b		730		b 352	
7. after uptake 1 hour	a		724 730	355	a 346 347	246
rinsed	b		735		b 348	
8. after uptake 20 hours	a		717 714	339	a 347 349	268
rinsed	b		711		b 350	

It has been investigated in Exp. W 84 (Table VI) in parallel experiments (I) and (II) whether leaves cut to uniform width before uptake give better results than leaf segments which retain their natural width. During pre-treatment and uptake, the 4 leaf segments of (I) had their natural width of about 8 mm and had a length of 5 cm. After uptake each leaf segment is cut in two parts of 2.5 cm giving 8 segments of 2.5 cm length per series.

The leaf segments of (II) have been cut at the beginning of the experiment to a width of 4 mm and a length of 2.5 cm.

The total area of series (II) is exactly 8 cm², that of series (I) is about the same but of course much more variable. In (I) and in (II) the uptake had a duration of 24 hours in the light and the external

solution was a 2.365 mM potassium chloride solution labelled with radioactive chloride 36, and with added calcium sulphate. After uptake, the series were rinsed for 5 minutes, one hour or twenty hours in 1 mM calcium nitrate solution. The results of this experiment give some useful information.

The data of (I) are quite unsatisfactory. There is a considerable loss of chloride during the first hour of rinsing, but this is only an apparent loss, and is not in accordance with the determinations of the increase of the chemically estimated chloride and the uptake of labelled chloride, which are equal. These data are, however, unreliable since the reference quantity, the length of the leaf segments, is not adequate. The increase of the chemically estimated chloride is obtained by the difference of the values B and A. As A is variable here, no conclusion about leakage can be drawn by comparing B–A with C. In the second place, the making of a fresh cut after uptake will have increased the variability of the data. It is clear that one cannot expect reliable data from experiments of this type. Also, an attempt to use as reference quantity the fresh weight of a series of leaf segments gave no better results. These objections are not valid with respect to our normal method when good material has been used. When the leaves are cut to a standard size, the amount of chloride in A is the same in all series.

The data of (II) are more satisfactory. After 5 minutes and 1 hour rinsing the same amount of chloride was found. The difference between increase of chemically analysed chloride and uptake of labelled chloride is not significant, showing that there is no leakage. After 20 hours rinsing there is a small decrease of the chloride content. Since here the increase of the chemically estimated chloride is smaller than the uptake of labelled chloride, it is possible that in these 20 hours there has been a small leakage, but it is slight in relation to the total amount of chloride present per leaf series (16 and 730 μg). In (II) also the use of the fresh weight as reference quantity does not improve the data. The error made in the determination of the fresh weight seems to be rather large in proportion to the accuracy of the chemical and radioactivity measurements per unit of surface area.

From this experiment it appears that leaf series made up by the standard method (8 leaf segments of 2.5 cm length and 4 mm breadth) give reliable results. All series have the same leaf area. If the leaf segments are not cut to uniform width the results are more variable.

If the material is sufficiently resistant the cutting of the leaves to standard width will give a normal uptake, assuming that enough time is given for full restoration of the tissue after cutting.

Table VII refers to Experiment W 103 which investigates the effect of cutting the leaves to 4 mm width before or after uptake.

In Table VII (I) the leafsegments were cut to 2.5 cm length and had their natural width during pre-treatment and uptake. After uptake they were cut to 4 mm width but not rinsed in order to prevent a loss of cell contents by the second cutting. The control series was treated in an analogous way. The segments of 2.5 cm length were cut to

TABLE VII

Influence of cutting the leaf segments to 4 mm width before or after uptake of chloride.

I. Leaf segments cut to 2.5 cm length and of natural width absorb chloride after the pre-treatment. After uptake they are cut to 4 mm width, no rinsing after second cutting.

II. Leaf segments of 2.5 cm length and 4 mm width absorb chloride after pre-treatment.

All series have been pre-treated in the light in 1 mM calcium nitrate solution for 24 hours. Uptake for 23 hours in the light from 1.5 mM potassium chloride solution, labelled with Cl 36 and containing calcium sulphate. Exp. W 103.

	Volhard estimations in μg chloride per series			Activity μg chloride/series measurement	
	before uptake A avg	after uptake B avg	Flux total Cl B–A	Flux labelled Cl C	avg
I A Cl content per series. After pre-treatment cut to 4 mm width. No rinsing after cutting	245 245 245				
I B. Cl content per series. After pre-treatment and uptake cut to 4 mm width. No rinsing after cutting.		497 504 511	259	250 260	255
II A. Cl content per series before uptake	220 213 206				
II B. Cl content per series after 23 hours uptake and fast rinsing.		525 518 516 504 518	303	309 305 292 308	304

4 mm width after the pre-treatment and not rinsed after this second cutting. In (II) all series were cut to 4 mm width and 2.5 cm length before the pre-treatment. This is the standard way of treatment. The control series II A were analysed after the pre-treatment, the series II B had an uptake period of 23 hours, and were rinsed after the uptake during some minutes.

In both experiments flux of total chloride ions (B–A) and flux of labelled chloride ions were equal. There was no leakage during uptake.

The amount of chloride present in the material before uptake was in (II A) about 30 μg smaller than that found in (I A). This could be expected since in (I A) leakage after the second cutting was prevented. The chloride uptake in (II B) is higher than in (I B).

This may be the result of the lower amount of chloride in the cells of (II A) than in those of (I A). It is not likely that it is caused by a loss of cell contents in I B after the second cutting, since B–A = C. It seems that both methods give reliable results, but that the uptake is larger in (II) than in (I). The larger uptake will chiefly be the

result of the lower initial chloride content in (II) which seems to bring about a higher uptake.

The method of determining uptake in material which is injured as little as possible seems to give workable results, provided that loss of chloride by rinsing after cutting the leaves has been prevented. Otherwise, variable results can be expected. The standard technique of cutting the leaves into segments of uniform dimensions, followed by a rather long period of pre-treatment, gives reliable results. It may be presumed that they do not deviate essentially from results obtained with intact leaves. The amount of chloride ions absorbed seems to depend on the quantity of ions which have remained in the cells after the cutting of the leaves and the preparation of the series. It may be that a lower osmotic value of the vacuole increases the amount of chloride ions absorbed.

Finally, we have examined the influence of making only one transverse cut through a leaf segment without further uptake. When translocation is being studied, it is a common procedure to cut leaf segments, 7.5 cm long for example, into three parts 2.5 cm long after translocation has taken place; chloride can then be estimated separately for each of the three parts. On account of this it would be interesting to find out what was, in fact, the effect of cutting the leaf segments after uptake had taken place.

In experiment W 113, (Table VIII) the most simple case is analysed; leaf lengths of 5 cm are divided into two equal segments of 2.5 cm. From Table VIII it appears that leaf segments of 5 cm length and

TABLE VIII

Influence on the chloride content of making a fresh cut.

In a first cutting the segments of series A, C, D were 5 cm long, those of series B were 2.5 cm long. The width of all leaf segments was 4 mm.

After 24 hours rinsing in calcium nitrate, the segments of series C and D were cut into two parts of 2.5 cm. C was not rinsed again; D was rinsed for 5 min. Determinations of C and D are in quadruplicate. Exp. W 113.

	μg Cl/series of 5 cm	μg Cl/series of 2.5 cm length
A. Leaf segments of 5 cm length and 4 mm width, rinsed in calcium nitrate 24 hours . . .	994 1005 1015	503
B. Leaf segments cut at 2.5 cm length and 4 mm standard width; after cutting 24 hours rinsed in calcium nitrate		a 462 469 b 476
C. Leaf segments of 5 cm length and 4 mm width, after 24 hours rinsing each segment is cut into two parts of 2.5 cm, and analysed; not rinsed again.		a 454 b 454 461 c 469 d 469
D. Leaf segments first cut at 5 cm length and 4 mm standard width, after 24 hours rinsing each segment cut in two parts of 2.5 cm, then 5 minutes rinsed and analysed. . .		a 440 b 436 445 c 451 d 451

4 mm width, which were immersed for 24 hours in calcium nitrate, contain 1005 μg Cl i.e. 503 μg Cl/ series of 2.5 cm.

The 5 cm leaf lengths treated in calcium nitrate for 24 hours were cut into two equal parts of 2.5 cm.

After being rinsed quickly they contain 461 μg Cl and after 5 minutes rinsing, 445 μg Cl; the fast rinsed series have lost 41 μg Cl, those rinsed for 5 minutes 60 μg/ series.

Leaf segments of 2.5 cm length after staying for 24 hours in calcium nitrate contain 469 μg Cl. In relation to the leaf segments of 5 cm length they have lost 34 μg Cl/ series of 2.5 cm.

The conclusion of this experiment is that cutting leaf lengths after uptake can give rise to large losses of chloride, particularly if the leaf series are rinsed after cutting.

This is an important practical result, which gives an acceptable explanation for results obtained in earlier experiments on translocation. It can be concluded that the results of cutting and handling the leaf segments depend largely upon the resistance of the material used.

With material of good quality, recovery after cutting is rapid and complete and the capacity for active uptake is high. During uptake there is no loss of chloride by exosmosis or exchange; after uptake the chloride absorbed remains unchanged for several hours. The removal of oxygen inhibits further uptake, but does not produce exosmosis of substances accumulated before. Metabolic inhibitors involved in active uptake (ARISZ, 1958) such as potassium cyanide, uranyl nitrate, arsenate, azide, and 2–4, dinitrophenol cause leakage only in high toxic concentrations, particularly in the case potassium cyanide and dinitrophenol.

With bad material, reliable results cannot be obtained. In such material leakage appears during uptake, and even during the preparation of the leaf series and the pre-treatment before uptake. The resistance to cutting of such material is poor and the power of recovery small. This gives rise to a large variation in the chloride content of the series at the end of the pre-treatment, when uptake is about to begin. When the initial chloride content (in the Tables A) is variable, there is no point in comparing the uptake of different series.

Material of intermediate quality can be used for experiments, provided that the plasma membrane remains impermeable during uptake. By changing the external solution or by handling the material, the permeability may be increased.

In preparing and treating the material, the transfer of all leaf segments together into the same solution has disadvantages, since, by the presence of a few leaking leaf segments, ions will be exuded into the external solution, and then they can be accumulated subsequently by other more resistant series (cf. Table X 4).

CHAPTER VI

LEAKAGE AND EXCHANGE OF CHLORIDE IONS AFTER UPTAKE OF LABELLED CHLORIDE IONS

It has been shown in Chapter III that during active uptake of labelled chloride in resistant material, no leakage of unlabelled chloride from the tissue occurs. This means that labelled chloride ions in the external solution are not exchanged for unlabelled ions in the tissue. If chloride ions cannot pass passively through the plasma membrane, neither passive loss nor passive uptake of chloride ions can be expected; neither will exchange of labelled with unlabelled chloride ions through the membrane occur. Loss and exchange of chloride ions are only possible if the plasma membrane has become permeable to chloride ions, or to pairs composed of chloride anions and cations such as K, Rb, Ca. When the material has already suffered a fairly heavy loss of chloride ions during uptake, then active uptake and passive loss or exchange cannot be identified, because different series will react differently with regard to leakage. This makes a comparison of their behaviour during rinsing impossible.

If the material has not displayed exchange or loss during uptake, it may be expected, that all series possess about the same amount of labelled and unlabelled chloride ions at the end of the uptake period. It can then be investigated whether by transferring the leaf series into solutions of different composition ion exchange, ion uptake or loss of ions occurs.

If exchange occurs between unlabelled chloride ions in the external solution and the previously absorbed labelled chloride ions in the tissue, the amount of labelled chloride in the tissue will be reduced and a similar quantity of unlabelled chloride ions will be absorbed from the external solution. The amount of chemically estimated chloride will then remain the same, but the amount of labelled chloride will decrease, i.e. B–A will be greater than C.

Three experiments, which fulfil the requirement that leakage does not occur during uptake, will be discussed here. The material used, though sufficiently resistant during uptake, was rather sensitive to changes in the external solution, and was, therefore, suitable for determining whether changes in permeability occur when the material has been immersed after uptake in rinsing solutions of different composition.

In each experiment the influence of a change of the uptake solution for a rinsing solution without chloride ions was investigated, and likewise the effect of a change for a solution containing unlabelled chloride ions was studied. In the latter solution an exchange of labelled chloride for unlabelled chloride can be expected if the plasma membrane becomes permeable to chloride ions.

In Table IX rinsing was effected by immersing the leaf series in a calcium nitrate solution during periods of different length (series 2,

TABLE IX

Leakage and exchange during rinsing after uptake.

Pre-treatment for 24 hours in 1 mM calciumnitrate solution in the light. Uptake for 24 hours in the light from a 1.5 mM potassium chloride solution, labelled with Cl 36 and containing calcium nitrate.

2–5 rinsed in 1 mM calcium nitrate fast, 5 min., 1 hour, 24 hours;

6–8 rinsed in 1 mM potassium chloride (unlabelled) containing calcium nitrate for 5 min., 1 hour and 24 hours. Exp. W 111.

	Volhard estimations in μg chloride per series			Activity measurements μg Cl/series Flux labelled Cl	
	before uptake	after uptake	Flux total Cl		
	A avg	B avg	B–A	C avg	
1. Cl content of material . . . a	504 508				
b	511				
2. after uptake fast rinsing . . a		703 700	192	a	203	198
b		696		b	192	
3. after uptake 5 min. rinsing . a		696 693	185	a	198	191
b		689		b	183	
4. after uptake 1 hour rinsing a		685 688	180	a	175	175
b		692		b	175	
5. after uptake 24 hours rinsing a		717		a	205	
b		440		b	118	
6. after uptake 5 min. a		689 686	178	a	177	176
KCl + Ca(NO₃)₂ . . . . b		682		b	174	
7. after uptake 1 hour a		689 689	181	a	177	173
KCl + Ca(NO₃)₂ . . . . b		689		b	168	
8. after uptake 24 hours a		696 703	195	a	177	176
KCl + Ca(NO₃)₂ . . . . b		710		b	174	

3, 4, 5), while in a calcium nitrate solution to which was added 1 mM unlabelled KCl (series 6, 7, 8) exchange could be studied.

The effect of KCl supply can be of two kinds, an effect brought about by the potassium ions and one brought about by the chloride ions.

The potassium ions may enhance the permeability of the plasma membrane and the addition of unlabelled chloride ions may involve an exchange of labelled chloride ions from the tissue for unlabelled ions from the external solution.

Both these effects have been studied in Table X and Table XI separately. In Table X potassium, calcium and sulphate ions were present in all experiments; in 5 and 6 unlabelled chloride ions were also added. In Table XI no potassium ions were added at all. Calcium and nitrate ions were supplied to all series, while in 5 and 6 unlabelled chloride ions were provided by addition of calcium chloride.

It might be expected that the results of these prolonged experiments would be more variable than those reported in Chapter III for chloride uptake. Some series have shown an incidental increase of permeability which caused leakage. Some clear cases of leakage are

found, in Table IX 5b (24 hours rinsing), Table X 6a, Table XI 3b; but they will not be discussed further here.

First, we shall consider the influence of being immersed in a rinsing solution, which does not contain any chloride. Chloride exchange is thus excluded. (Table IX 2, 3, 4, 5, Table X 2, 3, 4, Table XI 2,

TABLE X

Leakage and exchange during rinsing after uptake.

Pre-treatment for 24 hours in 1 mM potassium sulphate and calcium sulphate in the light. Uptake for 16 hours in the light from a 1.65 mM potassium chloride solution, labelled with Cl 36 and containing calcium sulphate.

2, 3, 4 rinsed in 1 mM potassium sulphate and calcium sulphate for 2 min., 1 hour, and 24 hours.

5, 6 rinsed in 2 mM unlabelled potassium chloride solution, containing calcium sulphate for 1 hour and 24 hours. Exp. W 90.

		Volhard estimations in µg chloride per series			Activity measurements µg Cl /series		
		before uptake A avg	after uptake B avg	Flux total Cl B–A	Flux labelled chloride C avg		
1. Cl content of material . . .	a	454 451					
	b	447					
2. after uptake 2 min. rinsing in	a		767 760	309	a	322	312
K₂SO₄ + CaSO₄	b		753		b	302	
3. after uptake 1 hour rinsing in	a		760 763	312	a	328	321
K₂SO₄ + CaSO₄	b		765		b	314	
4. after uptake 24 hours rinsing	a		817 814	363	a	314	
in K₂SO₄ + CaSO₄ . . .	b		811		b	288	
5. after uptake 1 hour in	a		740		a	280	
KCl + CaSO₄	b		735 739	288	b	283	286
	c		742		c	294	
	d		738		d	287	
6. after uptake 24 hours in	a		902		a	260	
KCl + CaSO₄	b		930 930	479	b	287	272
	c		944		c	270	
	d		944		d	270	

3, 4.) In these experiments, the differences between the increase of the amount of chemically estimated chloride ions and the amount of labelled chloride ions are small, although the variability is larger here than in Table I. This means that no appreciable leakage has occurred.

In one exceptional case, Table X 4a and b, there is a curious increase of chemically estimated chloride during the 24 hours' rinsing period in a solution, which was assumed not to contain any chloride (increase from 309 µg till 363 µg).

In order to understand this result, we have to realize that all the series shown in this table were put together in one large trough during the rinsing period. There may have been a leakage of chloride ions from the cells of some of the leaf segments of these series, and it

can then be understood that some of the other series with active leaf segments will have absorbed some of these chloride ions during their 24-hour immersion in this solution.

In Table IX 5, a during a 24 hours' rinsing period, a similar though smaller increase was observable. In Table IX 4 during a rinsing period of one hour there is a drop in labelled chloride. The cause of this loss is not known. We shall return to this point later.

The variability of these data permits only tentative conclusions. The impression one gets is that the impermeability of the plasma membrane is usually maintained during the rinsing period, and that in the absence of potassium ions and in the presence of calcium ions, changes are small (Table XI).

TABLE XI

Leakage and exchange during rinsing after uptake.

Pre-treatment for 24 hours in 1 mM calcium nitrate solution in the light. Uptake for 21 hours in the light from a 1.57 mM potassium chloride solution labelled with chloride 36 and containing calcium sulphate. 2, 3, 4 rinsed after uptake, in 1 mM calcium nitrate for 2 min., 1 hour and 4 hours. 5, 6 rinsed after uptake, in calcium chloride (unlabelled) containing calcium nitrate for 1 hour and 4 hours. Potassium ions are absent. Exp. W 94.

		Volhard estimations in μg chloride per series			Activity measurements μg Cl/series Flux labelled chloride	
		before uptake	after uptake	Flux total Cl		
		A avg	B avg	B–A	C avg	
1. Cl content of material . . .	a	312 309				
	b	306				
2. after uptake 2 min. rinsing in	a		612 612	303	a	305 300
Ca(NO₃)₂	b		611		b	295
3. after 1 hour rinsing in	a		611	302	a	295 295
Ca(NO₃)₂	b		589		b	273
4. after 4 hours rinsing in	a		614 613	304	a	313 312
Ca(NO₃)₂	b		611		b	310
5. after 1 hour in	a		624		a	298.
CaCl₂ + Ca(NO₃)₂ . . .	b		618 628	319	b	305 299
	c		643		c	299
·|	d		627		d	295
6. after 4 hours in	a		636		a	301
CaCl₂ + Ca(NO₃)₂ . . .	b		618 633	324	b	291 305
	c		636		c	312
	d		643		d	317

We have now to consider the influence of a rinsing solution containing unlabelled chloride ions. Since, in these experiments, unlabelled chloride ions were present in the external solution and could be actively absorbed, there would be no point, at this stage, in comparing the increase of the chemically estimated chloride ions with the absorbed labelled chloride ions.

In Table IX series 6, 7, 8 where KCl was supplied, there was only a slight increase of chemically estimated chloride ions, indicating that the power of active uptake was reduced. The amount of labelled ions had dropped from 198 μg to 175 μg. It is surprising that the same drop occurred in the rinsing solutions both with (6, 7, 8) and without (4) chloride ions. This indicates that the effect is obviously not a specific result of the addition of KCl and is, therefore, not an exchange. It may, in fact, be due to an increase in permeability of the peripheral membrane giving rise to exosmosis.

In Table X 5 we find after one hour's rinsing the same decrease in labelled chloride ions as in chemically estimated chloride. The decrease of labelled chloride ions indicates that labelled chloride ions have either been lost or exchanged. In the first case, the amount of chemically estimated chloride ions would have decreased; in the second case, it would not have changed. The decrease to 288 μg suggests that there has been a leakage, which may have been partly compensated for by an active uptake of unlabelled chloride ions during a one hour rinsing period in unlabelled potassiumchloride. Table X 6 shows that during a 24-hour rinsing period there was a high increase of chemically estimated chloride ions, while labelled ions decreased rather more than in Table X 5. These data do not indicate an exchange of labelled for unlabelled chloride ions; they can also be interpreted by leakage of labelled ions and active uptake of unlabelled ions.

In Table XI 5, 6 the external solution contained calcium chloride and calcium nitrate. No potassium ions were present during rinsing. Active uptake of unlabelled chloride ions was slight, probably on account of the accompanying calcium ions. Neither leakage nor exchange of chloride ions was found.

The general conclusion of these experiments is that during the rinsing period the impermeability of the plasma membrane to chloride ions is, usually maintained. Exceptions may arise when leakage takes place for no apparent reason.

The quantity of ions lost is very variable, and it may be rather large; for example in Table IX 5b it is about 80 μg Cl from the 200 μg Cl, in Table X 6 it is about 50 μg Cl from the 300 μg Cl, and in Table X 3b it is about 27 μg Cl from the 300 μg Cl. Such large quantities must partly be derived from the vacuole compartment. Rather more order in the pattern of leakage is found in Table IX 6, 7 and 8 where in the presence of KCl a fast but small decrease from 198 μg to about 175 μg occurs. The same quantity of chloride ions is lost in Table IX 4 after one hour rinsing in a solution without any potassium chloride. It may be that this rather small decrease of about 20 μg is caused by a change in permeability of the outer plasma membrane, but it is curious, that the effect of immersion for 5 minutes, one hour and 24 hours in potassium chloride and calcium nitrate is exactly the same as immersion for 1 hour in calcium nitrate alone. This may point to the presence of a small "compartment" containing this small amount of labelled chloride ions. The data are

insufficient to identify the compartment with certainty as the plasma compartment.

Also, as shown in Table X 5 after one hour's stay in a rinsing solution with potassium chloride a decrease of about 26 μg labelled chloride occurs. If there is a plasma compartment into which ions from the external solution are activily introduced, it seems likely that it contains only a rather small quantity of chloride ions and that by injury of the plasma membrane these ions are given off to the external solution. In the case of more severe injury exosmosis through the tonoplast also ensues.

In Table XI the presence of calcium ions seems to prevent changes and ruptures in the membranes, and therefore prevents exosmosis.

The data obtained are not consistent with the view that as the permeability of the peripheral membrane increases, the opportunity arises for exchange of ions between plasma and external solution.

CHAPTER VII

LEAKAGE CAUSED BY PLASMOLYSIS

ARISZ and VAN DIJK (1939), who investigated the influence of sucrose on the uptake of 1/80 M asparagine, showed that uptake of asparagine is accompanied by an increase in the osmotic value of the vacuole sap. If sucrose is added to the asparagine solution the uptake of asparagine increases, but when the sucrose concentrations produce plasmolysis, a considerable decrease of the asparagine uptake occurs. Repeating these experiments with chloride ARISZ and SOL (1956) found a similar result. A 0.24 M sucrose solution increased the chloride uptake from a 1 mM KCl solution, over a period of 24 hours, from 245 μg to 391 μg; addition of 0.32 M sucrose, a concentration which produces plasmolysis, gave an uptake of only 184 μg.

As a result of plasmolysis the plasmatic connections between the living protoplasts are disrupted and the protoplasm becomes separated from the cell wall. In this way leakage can arise and active uptake will be reduced.

It was interesting to examine whether, in such experiments, a leakage of the chloride absorbing cells could be demonstrated. The method described in Chapter III was used to compare the uptake of labelled chloride ions with the increase of the chemically estimated chloride ions. There was a 24 hour's uptake period from a 1.44 mM potassium chloride solution, labelled with Cl 36 to which was added both sucrose in different concentrations and calcium sulphate.

Table XII contains the results of Experiment W 109. It appears that addition of 0.2 M sucrose increases chloride uptake from 327 to 484 μg labelled chloride. The increase of the chemically estimated chloride is just as large as the uptake of labelled chloride even without addition of 0.2 M sucrose. This proves that no leakage occurred during the uptake in both cases.

TABLE XII

Leakage caused by plasmolysis.

Influence on chloride uptake of the addition of sucrose to a chloride solution. Pre-treatment 24 hours in the light in 1 mM calcium nitrate solution. Uptake during a period of 24 hours in the light from 1.44 mM potassium chloride solution, labelled with Cl 36, containing calcium sulphate and sucrose in different concentrations.

In 4 with 0.4 M sucrose leakage caused by plasmolysis is found, since B–A < C. Exp. W 109.

		Volhard estimations in μg chloride per series			Activity measurements μg Cl/series Flux labelled chloride	
		before uptake A avg	after uptake B avg	Flux total Cl B–A	C	avg
1. Chloride content of material	a	234 231				
	b	227				
2. 24 hours uptake from labelled KCl with calcium sulphate	a		561 561	330	a 331	327
	b		561		b 323	
3. 24 hours uptake from labelled KCl with calcium sulphate and 0.2 M sucrose . . .	a		710 716	485	a 484	484
	b		721		b 484	
4. 24 hours uptake from labelled KCl with calcium sulphate and 0.4 M sucrose . . .	a		273 273	42	a 102	101
	b		273		b 99	

If the sucrose concentration is raised to 0.4 M, a concentration which causes plasmolysis, the uptake of chloride is greatly reduced. The increase in the amount of chemically estimated chloride is lowered from 485 to 42 μg and the uptake of labelled chloride is 101 μg instead of the 484 μg, which is present after 0.2 M sucrose has been added.

The large difference between the increase of chemically estimated chloride and the uptake of labelled chloride proves that there is a leakage in the osmotic cell system. Probably both the plasmalemma and the tonoplast are injured. It is likely that under these conditions the active uptake process is greatly reduced. (Confer STADELMANN, SCHMIDT).

CHAPTER VIII

INFLUX AND EFFLUX OF ASPARAGINE

A. *Active uptake of asparagine*

During the first years of the investigation on uptake by *Vallisneria* leaves, the different behaviour of asparagine and caffeine in the processes of uptake and translocation was investigated. ARISZ and OUDMAN (1937, 1938) found that the absorption of asparagine is an active irreversible process connected with respiration, while that of caffeine is a diffusion process which is unrelated to respiration. It was

assumed, that the dipolar ions of asparagine are actively absorbed
as the plasma membrane does not permit these ions to pass passively.
The uptake of asparagine leads to an accumulation of asparagine in
the vacuoles, and in these uptake and secretion processes, free energy
potentials are used. The similarity between the uptake of asparagine
and the active uptake of salts was ascribed to the electric charge of
their ions, as both are electrolytes. Data about the accumulation factor
of asparagine (the relation between the concentration in the leaf and
in the external solution) can be found in ARISZ, 1943. In concentrations
of 1/20–1/160 M asparagine the accumulation factor increased from
1.3 to 5.3 (Table XIII). The asparagine absorbed is mostly accumu-

TABLE XIII

Accumulation of asparagine in 48 hours.

From ARISZ 1943 p. 641.

M conc. asparagine in medium	Nitrogen increase μg/series	M conc. asparagine in tissue (calc.)	Accumulation-factor
0.05	183	0.065	1.3
0.025	178	0.063	2.5
0.0125	139	0.049	4.0
0.00625	93	0.033	5.3

lated in the vacuoles and consequently the osmotic value of the cell
sap is enhanced.

This was verified by ARISZ and VAN DIJK, 1939 who determined
the osmotic value at limiting plasmolysis, before and after asparagine
absorption.

The relationship between active uptake of asparagine and respira-
tion appears when the leaf series are placed, during uptake in the
dark, in a McIntosh and Fildes anaerobic jar. Uptake is then greatly
depressed. Table XIV gives details of the influence of oxygen with-

TABLE XIV

Exp. VW 133. Influence of oxygen withdrawal on asparagine uptake.

Uptake from 1/40 M asparagine solution in the dark during a period of 22 hours.
After 22 hours oxygen was withdrawn from B, while series A remained under
aerobic conditions.

Duration of uptake	A Nitrogen increase in aerobic conditions in μg N/series	B Nitrogen increase oxygen withdrawn after 22 hours uptake
22 hours	75	75
26 hours	85	77
30 hours	93	74

drawal from the external solution. During 22 hours the leaf series had absorbed asparagine from a 1/40 M solution; subsequently oxygen was withdrawn by passing purified nitrogen gas through the solution. Uptake was then stopped, but exosmosis of asparagine did not take place. When oxygen was provided uptake continued. This experiment proves that active uptake in the dark requires oxygen, and that the withdrawal of oxygen does not provoke exosmosis although it stops active uptake. It will also be clear that exosmosis did not occur before the withdrawal of oxygen during the uptake of asparagine. In this way the impermeability of the plasma membrane to asparagine can be demonstrated.

As this experiment had to be carried out in the dark to avoid oxygen formation by photosynthesis, it may be asked whether there is a difference in the uptake of asparagine in the light and in the dark. Table XV shows that there is a marked difference, though a considerable uptake is found in the dark. Some more recent experiments have shown the influence of inhibitors on uptake. Cyanide

TABLE XV

Exp. Z 14. Uptake of asparagine in light and in darkness.

Uptake from a 10 mM asparagine solution during 24 hours.

	μg N/series
Uptake in the light, aeration with CO_2-free air	256
Uptake in the dark, aeration with CO_2-free air	136
Uptake in the light, aeration with air	239
Uptake in the dark, aeration with air	126

(Table XVI) and uranyl nitrate (Table XVII) inhibit asparagine uptake as well as chloride uptake (ARISZ, 1958).

Since the 1939 publication of ARISZ and VAN DIJK, the study of asparagine uptake has been continued, with particular reference to exosmosis of previously absorbed asparagine. The problem of in-

TABLE XVI

Exp. M 825. Influence of KCN on the uptake of asparagine.

Uptake of a 10 mM asparagine solution at pH 6 during a period of 24 hours. Pre-treatment 24 hours in water in the light.

	μg N/series
After 24 hours asparagine uptake	249
— with addition of 3.10^{-5}M KCN	250
— with addition of 10^{-4}M KCN	98
— with addition of 3.10^{-4}M KCN	0

TABLE XVII

Exp. Z 3. Influence of uranyl nitrate on asparagine uptake.

24 hours pre-treatment in the light in water.
24 hours uptake of a 10 mM asparagine solution in the light.

	μg N/series
After 24 hours asparagine uptake	344
— with addition of 10^{-6} M uranyl nitrate	296
— with addition of 10^{-5} M uranyl nitrate	146

fection by microorganisms in experiments using asparagine also had to be assessed. A study was also made of the infiltration of the intercellular ducts in *Vallisneria* leaves, as these ducts are opened by cutting across the leaves and can become more or less filled up with drops of the external solution.

If asparagine is present in the external solution, an increase in the amount of asparagine in the tissue will be obtained by this infiltration, unless the asparagine is given off again from the ducts by rinsing for a long enough time.

The results of these experiments could not be published fully during the war. Only a short communication was published in 1943 in the Dutch language, (Verslagen Ned. Akad. Wet., 1943). Since this publication was difficult to obtain, a few data have been reproduced in a recent publication (ARISZ, 1958). The present investigation deals with identical problems with regard to chloride uptake. It considers the difficulties involved in experiments on uptake of chloride by leaves of *Vallisneria*. It seemed worthwhile to give here a more detailed report of the difficulties formerly encountered in studying asparagine uptake. These problems deserve our consideration the more, since by the influx and efflux theory of BRIGGS, HOPE and ROBERTSON, the physiological connection between active uptake and permeability of the plasma membrane has become a matter of general interest.

The result of the critical evaluation of the experiments on asparagine uptake was a corroboration of the results of ARISZ and OUDMAN. It will, of course, be necessary to extend the asparagine experiments with the help of new methods using tracers and chromatographic analysis of amino-acids. New data on the kinetics of uptake of amino-acids have been published by BIRT and HIRD, 1958.

I confine myself here to three points of practical interest; contamination with bacteria, infiltration of the air-ducts in the leaves with external solution and exosmosis of absorbed asparagine.

B.   *Contamination with bacteria*

Repeating some experiments of SCHWABE on the influence of amino-acids on the respiration of *Elodea* shoots, SCOTT RUSSELL 1932 found that the increase of oxygen consumption was for the greater part caused by bacterial contamination. By rinsing the shoots several times

in sterile water the growth of bacteria could be largely prevented, and a much smaller effect of amino-acids on respiration was found.

These data immediately posed the question whether in the process of asparagine absorption also the reliability of the results could be unfavourably influenced by bacterial contamination. There can be no doubt that during uptake of asparagine bacterial growth can occur, but the techniques used tended to prevent bacterial infection. The material was always carefully rinsed and it was usually pre-treated before uptake by remaining in water for 24 hours. This must according to the results of Scott Russell, have reduced infection by bacteria. However, in some experiments which were rather heavily infected by bacteria, asparagine breakdown was found and ammonia was produced. It could be shown that the leaves of *Vallisneria* are able to absorb ammonia from very low concentrations (Table XVIII).

TABLE XVIII

Absorption of ammonia in light and darkness.

Colorimetric nitrogen determinations with Nesslers reagent.

	μg N in the light	μg N in the dark
4 hours uptake of 0.5 mM NH$_4$OH	26	26
23 hours uptake of 0.5 mM NH$_4$OH	39	39
4 hours uptake of 0.1 mM NH$_4$OH	6	
23 hours uptake of 0.1 mM NH$_4$OH	9	

It appeared, by determining the ammonia concentration of the external solution with Nesslers reagent, that from 0.1 mM solution of ammonia, 9 μg was absorbed per series within 23 hours.

As asparagine uptake was determined by Kjeldahl measurements of the nitrogen, the presence of ammonia in the external solution could be the cause of inaccurate results. It seemed desirable there-fore, to assess the bacterial contamination by determining the ammonia content of the external solution; on this basis, experiments in which higher ammonia concentrations, higher than 0.1 mM, were obtained, were discarded. Such cases were exceptional, as generally no trace of ammonia was found. Two exceptions have to be made. Experiments of longer duration e.g. 48 hours often show an effect of contamination. Resistant material has to be used. If the material is less resistant and leakage of the cell contents occurs, this will have a favourable in-fluence on bacterial growth.

C. *Infiltration of the air ducts in the leaves*

Infiltration of the intercellular spaces is a common phenomenon in many plants, and will be discussed later. Here, we confine our-selves to a process occurring particularly in *Vallisneria* plants con-taining air ducts in their leaves, which are opened when the leaves

are cut across for obtaining leaf segments of standard size. The external solution will be sucked in by capillary forces or by suction. The phenomenon depends on respiration, which uses oxygen from the air ducts, whilst the carbon dioxide produced is dissolved and does not appear as gas in the air ducts. In the light oxygen consumption will be compensated for by oxygen formed in photosynthesis; in the dark therefore infiltration will be greater than in the light (Table XIX and Fig. 2).

When the external concentration is higher than 1/80 M, a correction

6   TABLE XIX

Infiltration of the airducts in light and in darkness during asparagine uptake: Infiltration is measured as an increase of fresh weight.

	Asparagine uptake in the light			Asparagine uptake in the dark		
	μg N	in mg	μg N	μg N	in mg	μg N
1/10 M	92	0	0	126	15	—42
1/20 M	82	3	—4	123	26	—36
1/40 M	82	4	—3	79	20	—14
1/80 M	53	3	—1	61	21	— 7
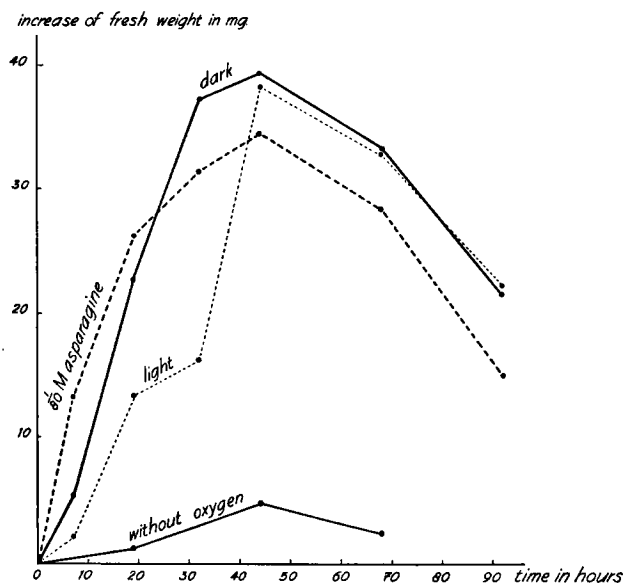
Fig. 2. Infiltration after cutting. The increase in fresh weight of leaf series consisting of 12 leaf segments of 8 mm length is measured. Each series has an initial weight of about 100 mg. The series were placed in water or in asparagine solution, in the light or in the dark, in aerobic or anaerobic medium. In aerobic experiments, the increase in weight amounts to 35 to 40 % of the fresh weight.

of the nitrogen increase per leaf series is needed in respect of the asparagine solution introduced into the air ducts during the uptake period. It may be assumed that the increase in weight gives an idea of the degree of infiltration. An increase of 1 mg from a 1/80 M asparagine solution causes an increase of about 0.35 μg N. The correction of the determination of the uptake will, therefore, only be of any significance when the solutions used have higher concentration than 1/80 M. Pronounced infiltration is only observable when uptake occurs in the dark, and when the external solutions have a concentration of about 0.1 and 0.2 M asparagine. The accuracy of the estimation of fresh weight per series is rather small.

This work did not examine whether the increase in fresh weight is caused by growth, by active uptake from the external solution or by infiltration; it provided only an upper limit for the infiltration.

D. *Exosmosis of asparagine*

The *Vallisneria* material used in the experiments of ARISZ and OUDMAN must have been fairly resistant. In describing experiments with leaves absorbing from asparagine solutions they remark "in no case was a decrease of the nitrogen content found within 72 hours although this was found in some case after 96 hours" (1938, p. 812).

ARISZ and VAN DIJK (1939) mentioned that "after 48 hours uptake from a 1/80 M asparagine solution, the increase of the osmotic value of epidermal cells was 0.047 M. After absorption the leaf segments were put into water for 4$\frac{1}{2}$ hours to allow exosmosis to take place. The osmotic value of the cell sap was then found to have fallen by 0.031 M. Chemical analysis by Sachse's method of amide nitrogen determination showed that during exosmosis asparagine in particular enters the external solution, indicating that most of the asparagine absorbed during the uptake period was not converted by cell metabolism.

In the experiments discussed by ARISZ, 1943, the material was less resistant and the amount of asparagine lost by exosmosis was rather variable, whilst in the second half of 1943 the material was of such good quality that practically no exosmosis could be detected.

Exosmosis of asparagine was determined by estimating the amount of nitrogen per leaf series before and after transfer of the series into a fresh solution. Table XX gives an example of a decrease in the nitrogen content in the first two hours after uptake amounting to about 30 μg N, whilst in the following 18 hours the amount does not show any further change. A decrease may point to leakage, but part of the loss is caused by loss of asparagine from the free space and from the infiltrated air ducts. The loss from the free space after uptake from a 1/40 M asparagine solution can amount to 5 μg N per series, and it is recorded in Table XX that the infiltration amounts to 5 μg N per series.

Moreover, during immersion in the external solution in these earlier experiments, the leaf series were sewn up in strips of tulle to

TABLE XX

Exp. VW 157. Time course of exosmosis of asparagine in water.

Leaf series absorbed asparagine in the light, from a 1/40 M solution, for 23 hours. Estimations in triplicate.

Duration of exosmosis after asparagine uptake	Nitrogen absorbed in μg	Infiltration in mg	correction in μg
0 hour	92	7	5
2 hours	59		
4 hours	63		
8 hours	63		
20 hours	60		

prevent them from being mixed up and from floating on the surface of the solution.

When exosmosis had to be investigated during periods of different length it was necessary to replace the strips of tulle by fresh ones directly after uptake. It seems that the decrease of the nitrogen content in the first two hours was the consequence of different factors. One of these may have been an increased permeability of the plasma membrane. This opinion is supported by experiments reported in Fig. 3, 4, 5 and in Table XXI. It is the change of the external solution, which suddenly alters the permeability of the plasma membrane.

In Fig. 3 several leaf series had absorbed asparagine from a 1/40 M solution for 24 hours. When the leaf series were transferred to fresh asparagine solutions of different concentration their permeability was changed. The new rate of uptake depended on the asparagine con-
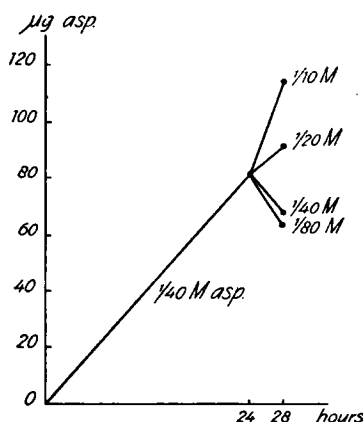

Fig. 3. Leakage after asparagine uptake. Absorption of asparagine from a 0.025 M asparagine solution over a period of 24 hours; thereafter, 4 hours in asparagine solutions of different strength. (From ARISZ, 1943, Fig. 4).

centration of the external solution. In 1/20 and in 1/10 M solutions uptake was continued, but in 1/40 and 1/80 M solutions exosmosis had begun. This gave the impression that the influence of active uptake processes had temporarily been reduced. It cannot be concluded whether there was a decrease of the active uptake, either together with, or instead of an increase in passive diffusion processes depending on the concentration difference between the tissue and the external solution.

The change in permeability of the plasma membrane seems to be reversible since, after some hours, active uptake is resumed.

This is shown in Fig. 4 where, during a period of 4 hours in water
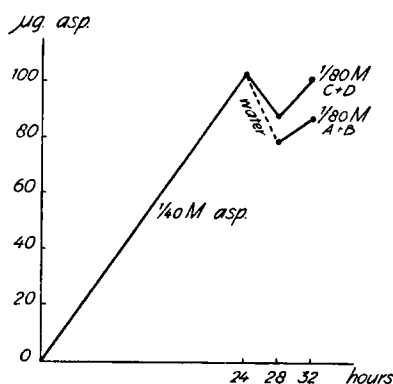
Fig. 4. Leakage and recovery after asparagine uptake. Absorption of 0.025 M asparagine solution during a period of 24 hours; thereafter A and B four hours in water, C and D in 0.0125 M asparagine solution. In the following 4 hours all series were in 0.0125 asparagine solution. (From ARISZ, 1943, fig. 2).

or 1/80 M asparagine solution, exosmosis occurred, while in the following 4 hours active uptake from a 1/80 M asparagine solution was resumed. Detailed understanding of the combination of active and passive processes, of influx and efflux cannot be gained from experiments of this type. The uniform behaviour of leaf series, which have absorbed asparagine for 4 and 24 hours from a 1/40 M solution, is rather unexpected. Fig. 5 and Table XXI give the report of two experiments. When the leaf series are transferred into water, 1/20 and 1/10 M asparagine solutions an identical change in permeability is found as in Fig. 3. The very similar reactions after 4 and 24 hours asparagine uptake in Experiment I are striking. In experiment II the differences are somewhat larger but still remarkably small.

Since the experiments on exosmosis gave the impression that the permeability of the plasma membrane was changed, it seemed of interest to discover whether the stability of the plasma membrane could be favourably influenced by addition of salts.

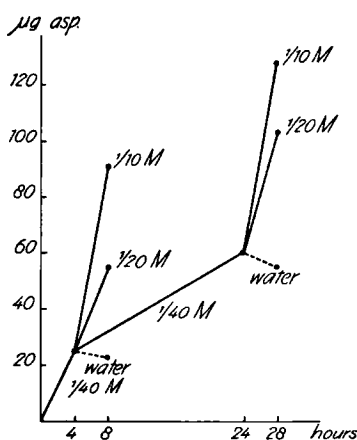Calcium sulphate, magnesium sulphate and potassium chloride

Fig. 5.   Influx and efflux of asparagine in solutions of different strength after 4
and 24 hours uptake from a 1/40 M asparagine solution.

TABLE XXI

Exp. I (VW 136), II (S 223). Passive asparagine uptake caused by changing the
external solution after 4 and 24 hours asparagine uptake.

   A. After 4 hours asparagine uptake from a 1/40 M solution in the dark, the leaf
series are transferred to a 1/10 or 1/20 M asparagine solution or to water, and the
nitrogen increase is measured after remaining for 4 hours in these solutions.

   B. The same is repeated after 24 hours uptake from the 1/40 M.

asparagine solution	μg N increase		change in μg nitrogen during second period	
	exp. I	exp. II	exp. I	exp. II
A 4 hours uptake from 1/40 M asparagine sol.	25	34		
— and 4 hours 1/10 M asparagine sol.	91	80	66	46
— and 4 hours 1/20 M asparagine sol.	55	65	30	31
— and 4 hours water	18	32	— 7	— 2
B 24 hours uptake from 1/40 M asparagine sol.	60	122		
— and 4 hours 1/10 M asparagine sol.	128	164	68	42
— and 4 hours 1/20 M asparagine sol.	103	134	43	12
— and 4 hours water	55	118	— 5	— 4

were added to the asparagine solution until a total salt concentration
of 0.01 M was attained. (Table XXII) The uptake of asparagine from
this balanced solution was greater than from a pure asparagine
solution. At pH 7 a nitrogen increase of 121 μg was obtained. After
a period of 4 hours in a balanced solution without asparagine, a loss
of asparagine from the tissue was not observed.

At a pH of 4.6 a somewhat lower quantity of nitrogen, 100 μg,
was found after uptake, while after a 4-hour period in balanced salt
solution without asparagine, a slight decrease had occurred. At this

TABLE XXII

Exp. S 220. Uptake of asparagine from a balanced solution containing, besides asparagine, potassium chloride, calcium sulphate and magnesium sulphate.
    Uptake in the light from 1/20 M asparagine solution during a period of 24 hours.

	N increase
A Uptake from asparagine solution at pH 4–5	90 μg
B Uptake from balanced asparagine solution at pH 4–5	109 μg
C Uptake from balanced asparagine solution at pH 7	121 μg
After uptake according to C rinsed for 4 hours in balanced salt solution	119 μg

lower pH the plasma membrane was apparently not completely impermeable to asparagine.

In Table XXIII potassium chloride together with calcium sulphate and asparagine were added, and the uptake of chloride and nitrogen estimated. The material was in good condition and the solution was balanced. Chloride and asparagine uptake are both high (15 μM Cl and 9.5 μM asparagine), and the experiment shows that the influence of pH on the uptake of chloride and asparagine is not significant.

TABLE XXIII

Exp. Z 21. Simultaneous uptake of 1 mM potassium chloride and 10 mM asparagine solution containing calcium sulphate.
    Estimations of chloride and nitrogen in μg /series.

	Increase of chloride μg Cl	Increase of nitrogen μg N
pH 5	536	274
pH 6	529	267
pH 7	525	258

When the influence of pH on asparagine uptake was investigated variable results were often obtained, especially at lower pH. With resistant material and in balanced solutions the influence of changes in pH are relatively small. Also, in these experiments the favourable influence of the addition of calcium to potassium chloride solutions has been found to have a favourable effect. In this respect the present research is in close agreement with the classical work on ion antagonism of OSTERHOUT, STILES, HOMÈS and others. Ion uptake is, like growth, sensitive to the ionic balance of the external solution.

DISCUSSION

A. *Influx and efflux of chloride and asparagine*

In *Vallisneria* leaves electrolytes are absorbed into the cytoplasm
by active processes using free energy potentials. Besides these active
processes passive fluxes, influx and efflux of electrolytes are found.
This depends on the permeability of the plasma membrane to these
electrolytes.

The term *permeability* of the plasma membrane is restricted in this
publication to those physico-chemical processes, which enable sub-
stances to pass through the plasma membrane without making use
of free energy potentials provided by cell metabolism. When metabolic
processes are involved the term *active uptake* will be used.

Changes in the permeability of the plasma membrane may occur
during the preparation and pre-treatment of the material and during
the uptake and rinsing periods preceding the determination of the
amount of electrolytes absorbed.

It has been shown in "Influx and efflux I" that in healthy, vigor-
ously growing *Vallisneria* plants the peripheral plasma membranes of
the leaf cells are impermeable to chloride ions. This means that
permeability is so low that exosmosis cannot be measured.

Material of this quality is called *resistant* if the permeability does
not change during the uptake. If, however, the permeability of the
plasma membrane increases during the manipulation and ions pass
the membrane passively, a *secondary* state of diminished resistance is
prevailing. The state of impermeability of the plasma membrane is
distinguished as a *primary* state.

The terms normal and abnormal will not be used, because the
state of increased permeability of the plasma membrane, which is
secondary in *Vallisneria* leaf cells, may, in cells of other plant species
be the normal state; and in *Vallisneria* also, plants which have cells
with increased permeability and leaves with infiltrated spots, this
condition can be more permanent. Such a longer lasting change of
the primary state can be the result of diseases or infections by viruses
or algae. Besides, it may be that not all ions behave in the same way
as chloride ions.

From an ecological point of view it will be interesting to consider
and to study these possibilities e.g. adaptations of water plants living
in an environment of periodically changing salt concentrations.

In "Influx and efflux I" the permeability to chloride ions of the
peripheral plasma membrane has been investigated by a method
which provides the opportunity of determining whether active uptake
and passive exosmosis of chloride ions occur side by side (ARISZ, 1963a).

It appeared that in resistant material during the active uptake of
chloride neither a passive loss of chloride ions through the membrane

nor an exchange of unlabelled chloride ions from the tissue with labelled chloride ions in the external solution occurred. This proves that in this material the peripheral plasma membrane is not permeable to chloride ions.

It may be emphasized that the presence of an active exchange process, which is independent of the permeability of the plasma membrane, is also excluded by these results.

COLLANDER (1939) has defended the view, that plasma membranes are non-permeable to all ions, to anions as well as to cations, to hydrogen ions and to hydroxyl ions. The results obtained from *Vallisneria* plants in "Influx and efflux I" are in complete agreement as to impermeability to chloride ions. There is only one limitation; the plants must be sufficiently resistant to changes in the permeability of the membrane so that a permanent impermeability of the membrane can be maintained.

The problem has not yet been studied sufficiently for ions other than chlorides. I am inclined to support the view of COLLANDER that plasma membranes exist which are impermeable to all ions. One must also consider whether such impermeable membranes change their structure later on and become more permeable. The conditions, which admit passage through the membrane, need not be quite the same for all ions. This means that the permeability has to be studied for each kind of ion or ion-pair, and that this has to be repeated for each kind of tissue separately.

In "Influx and efflux II" changes in the permeability of the plasma membrane to chloride ions have been studied using the new method described in "Influx and efflux I". This method compares the increase of chemically estimated chloride ions with the uptake of labelled chloride ions after uptake of labelled chloride ions. During uptake of labelled ions through a membrane with increased permeability there will be a leakage of unlabelled ions, which were already present in the tissue before uptake began, as well as a leakage of newly absorbed labelled ions.

The leakage of labelled chloride ions decreases the flux of total ions as much as that of labelled ions, but the leakage of unlabelled chloride ions decreases the flux of total ions but not that of labelled ions. During uptake therefore leakage manifests itself by a difference between the flux of total ions and that of labelled ions. If leakage occurs, the net flux of total ions is lower than the flux of labelled ions. (Chapter III).

In the case of uptake of labelled chloride ions by *exchange* with unlabelled chloride ions in the tissue, an increase of the influx of labelled ions may be expected, while the amount of total chloride ions will remain unaltered. The flux of total ions will become less than flux of labelled ions; the result is the same as when leakage occurs. Consequently, a comparison of the fluxes does not distinguish between exchange and a combination of leakage with active uptake.

Exchange can also be postulated, when after uptake of labelled chloride ions the tissue is placed in a solution with unlabelled chloride

ions and the permeability of the membrane has been increased by handling. In this case the amount of total chloride present in the tissue after uptake will remain unaltered, but the amount of labelled ions in the tissue will decrease. In this case also a discrimination between a combined process of leakage of labelled ions and active uptake of unlabelled chloride and an exchange of labelled with unlabelled ions from the rinsing solution could not be realized.

The data given in Chapter VI point rather to increased leakage and loss of chloride ions from the cytoplasm combined with active uptake of unlabelled chloride ions from the external solution, than to exchange.

In resistant plants chloride ions and asparagine once they have been actively absorbed are not exuded again unless permeability of the plasma membrane has been increased. When metabolism is reduced by the withdrawal of oxygen or by the application of substances which inhibit active uptake when supplied in appropriate concentrations, the level of the ions absorbed into the cell is not changed. This proves that a continuous supply of energy is not required for maintaining the level of absorbed ions in the cell and suggests that there is a general impermeability of the peripheral plasma membrane to non lipid substances. It is remarkable that the uptake of asparagine and chloride are similar in so many respects. According to ARISZ and VAN DIJK (1939) the active uptake of asparagine is connected with the polar structure of the dipolar ions. They react in the same way on potassium cyanide and uranyl nitrate and it is known (ARISZ, 1958) that these substances inhibit the first step of the uptake, which is the introduction of the ions from the external solution into the cytoplasm. Both the uptake of asparagine and that of chloride are increased in the light, and one gets the impression, that in the dark the uptake of asparagine is relatively stronger than that of chloride. It is likely that uptake in the dark depends on free energy potentials in store or newly formed in respiration, and that as a result of the absorption of asparagine the respiration is enhanced and more free energy potentials are made available. (cf. SCHWABE, SCOTT RUSSELL, page 30).

The mechanism by which these ions are introduced into the cytoplasm is insufficiently understood. The activity of the inhibitors mentioned indicates that energy-rich phosphates are involved in this process.

B. Ion antagonism and balanced solutions

During asparagine uptake as well as during salt uptake changes of the plasma membrane permeability can appear, which are presumably caused by the same factors. It is a common view that the ionic composition of the plasma membrane has a regulating influence on its permeability. The structure of the membrane can be influenced by several factors such as changes of heat and light, by electric stimuli and particularly by changes in the relative proportion of cations like calcium, magnesium, hydrogen, potassium and sodium in the external

solution. The importance of these cations is well known by the classical researches of LOEB, OSTERHOUT, STILES, HOMÈS and many others.

The membrane at its outer boundary is in balance with the relative concentration of the different cations in the external solution and in the free space. Hydrogen, potassium and calcium ions have to be present in the plasma membrane in definite proportions in order to maintain its primary state of impermeability. If the proportions of these ions are altered the membranes lose their stability and become more permeable. Univalent ions particularly are injurious and cause leakage, whereas the addition of divalent ions restores the primary condition of the membrane by their antagonistic action. Also without adding fresh salt ions, only by rinsing the tissue in calcium free solutions calcium ions will be removed from the membrane and the balancement disturbed. It is therefore possible to retain the stability of the membrane during pre-treatment, rinsing and exchanging periods and also during uptake itself, by using balanced solutions containing antagonistic ions.

STILES has ascribed ion antagonism to a mutual hindrance to the entrance of toxic ions into the cell. At present we know that the ideal constellation of the plasma membrane is that in which it is non-permeable to electrolytes. Permeability is increased by so-called toxic ions and the non-permeability is retained or restored by antagonistic ion action. This proves that at least smaller changes in the membrane structure are reversible. The balanced solution of OSTERHOUT, STILES, HOMÈS and others is essentially a solution, which maintains the non-permeability of the peripheral plasma membrane. The effect of a pre-treatment of *Vallisneria* leaves with salt solutions on uptake of chloride ions, studied by SOL, 1958 is an indication of the regulating influence of the ionic constellation of the plasma on the uptake of ions from the external solution. An identical influence of the presence of salts on waterpermeability has been discovered by IZ. DE HAAN (1933, 1935).

STILES (1924, p. 241) has indicated that the so called reversible changes in permeability may be attributed to reversible changes in molecular associations. This is an attractive hypothesis (Confer DAVSON and DANIELLI, 1952).

It may be expected that after the ions have passed the peripheral plasma membrane they cause identical changes in the cytoplasm and in the adjoining vacuole membrane, the tonoplast. A heavy loss of ions from the tissue indicates that not only the ions from the plasma compartment but also those from the vacuole are diffusing outwards through the tonoplast, the cytoplasm and the peripheral plasma membrane.

As a consequence of this exosmosis the osmotic value of the vacuole sap decreases.

This has been shown by ARISZ and VAN DIJK (1939) for exosmosis of asparagine, and has been corroborated by Miss VAN SCHREVEN and Miss VAN DER MOLEN (unpublished results, confer ARISZ, 1943, 1948 and 1960) for exosmosis of chloride.

The antagonistic effect of ions and the influence of balanced solutions on the permeability of the membrane can be better demonstrated in the active uptake of asparagine than in the uptake of solutions containing salts, because by using asparagine a specific effect of salt ions on the permeability of the membrane to asparagine is elucidated.

In experiments on asparagine uptake with balanced solutions no exosmosis of asparagine either during or after uptake was found. (Table XXII and XXIII). This experiment with asparagine proves that the potassium and calcium ions really act on the membranes and not on the binding of the ions to plasma molecules, since asparagine is present as a free amide in plasma and vacuole and not chemically bound.

HIGINBOTHAM, PRATT and FOSTER (1962) have investigated the influence of indole acetic acid and calcium on the absorption of potassium and rubidium by excised segments of etiolated pea epicotyl. Their conclusions are akin to the view given above; this is apparent from the following quotation: "potassium in the absence of calcium may tend to increase permeability by replacing calcium from rather specific sites in the membrane. Calcium seems to play a role normally as an ion antagonist and presumably it may act by occupying specific sites at protoplasmic surface to give the protoplast greater stability and greater selectivity".

C.   *Influence of cutting the leaves on leakage of chloride*

The results of experiments on uptake are often variable due to a change in the permeability of the plasma membrane of the leaf cells. In all quantitative experiments the material has to be cut to standard size. This made it desirable to know the effect of cutting on the course of the uptake process. It has been shown in Chapter V that cutting the leaves causes leakage of the cell contents. Some cells are entirely destroyed, in others, the plasma membranes have increased in permeability. After some time a recovery sets in, as leakage is reduced and the rate of uptake increases again. Resistant material displays a rapid and complete recovery, but material which is insufficiently resistant gives variable results. After recovery the original structure and non permeability of the plasma membranes is restored. Cell metabolism then produces once more the free energy potentials needed for the metabolic reactions involved in the uptake process. Only after a longer period of recovery does the capacity of the symplasm for ion migration return.

It is remarkable, that even after such severe injuries as cutting the leaves, quite normal results of ion uptake and transport can be obtained, when the material is sufficiently resistant.

The data obtained permit a better understanding of the draw-backs arising from the use of slightly resistant material. By cutting the leaves permeability changes and disorganisation of the cells is caused.

Only cells with the power for complete recovery will provide the

homogeneous series, which are an essential condition to obtain accurate results. When the amount of ions present in the tissue before uptake is variable, reliable data about uptake cannot be obtained.

If after uptake fresh wounds are made, a new loss of cell contents can be expected. Consequently the cutting of the leaves after uptake or after translocation has to be performed with precautionary measures to prevent loss of cell contents.

It has been found, that the quantity of chloride lost after cutting depends on the mutual distance of the cuts. Therefore short leaf pieces have a relatively lower chloride content than longer ones after pre-treatment. When the chloride content of leaves of different length is compared, this difference has to be kept in mind.

D.   *The ions in the symplasm and the peripheral plasma membrane*

The cytoplasm plays a central role in the physiology of the cell. It regulates uptake and secretion and is the path which enables substances and ion pairs to move rapidly. To think of uptake into the cytoplasm and secretion into the vacuole together with movement in the tissue, as one process of cell uptake, is an underrating of the regulating function of the plasma. Little is known about the inter-action between the different organelles and compartments in the cell. Energy-rich substances locally formed by photosynthesis and respiration have to migrate in the cellplasma to the places, where energy spending reactions occur. When there are processes occurring in tissues, intercellular translocation takes place through plasmatic connections like the plasmodesmata which unite the single cells to a symplasm. This makes us understand the remarkable uniformity of adjoining cells in a tissue.

The significance of the symplasm in the translocation of ions has been discussed in several publications (Arisz, 1945, 1956, 1960b, 1963b). The discussion is confined here to the question of whether the ions in the plasma are chemically bound or free or at least freely moving, but prevented from escaping from the cytoplasm into the outer solution, because they cannot pass through the peripheral plasma membrane.

It has been shown in preceding publications, that the entrance of chloride ions into the plasma is a biochemical process, which can be separated from the secretion into the vacuole and the migration in the symplasm by comparing the result of application of an inhibitor like potassium cyanide, arsenate, or uranyl nitrate, to the zone of entrance and to the contiguous transporting zone where secretion in-to the vacuole takes place (Arisz, 1953, 1958).

The results point to the presence of a peripheral membrane, which has to be passed only once in the entrance zone to introduce the ions into the symplasm and once in the symplasm these ions continue moving over fairly large distances (Arisz, 1963b).

Besides it has been shown that the chloride and asparagine ions in the symplasm migrate fairly rapidly and independently in a

diffusion-like process, as a consequence of concentration gradients (ARISZ 1960a and b, 1963b). To prevent their exosmosis a non-permeable plasma membrane is necessary. Exchange of chloride ions between symplasm and external solution has not been found. The amount of movable ions in the plasma-pool seems to be considerable. This can be concluded from current experiments on ion migration not yet published.

On the ground of these results the opinion of some investigators, that the peripheral membrane is permeable and that the ions in the plasm are irreversibly bound to prevent their escape to the outer medium, (TROSHIN) can be rejected. The evidence obtained by the experiments on ion migration in the symplasm and the changes in membrane permeability studied in this publication justify the view, that the ions in the plasma are able to migrate in a diffusion like process. They retain their specific qualities during this migration and do not diffuse to the outer solution because the symplasm is surrounded by a membrane, which in its primary state is non-permeable to ions.

The possibility, that the migration of ions and ion pairs is localized in special plasmatic ducts, has not been considered here by lack of data.

E. *Uptake of ion pairs*

As it is a result of the present investigation that active and passive exchange of chloride ions through non-permeable membranes is impossible, the active passage of these ions through the plasma membrane in its primary state must occur as ion pairs, because electrostatic balance has to be maintained.

When leaf segments are placed in a salt solution diffusion of the salt ions into the free space of the tissue begins. In *Vallisneria* cells the free space is small, only 5–7 % of the volume (KYLIN 1960, WINTER 1961). As far as the tissue is not separated by non-permeable membranes from the medium exchanges may occur between ions of the tissue and ions of the external solution. In some objects, but not in *Vallisneria* leaves exchange of anions has been found, but the exchangeable anions seem to be confined to the cell walls and are not derived from the symplasm. When studying the results of active uptake into the plasma, the ions introduced by diffusion and exchange have to be subtracted from the total amount absorbed. This applies particularly in experiments of short duration, when labelled ions are absorbed from solutions of high concentration. Only the results of active ion uptake will be considered here. However, separation of active and passive uptake is difficult, when undissociated molecules of weak acids and bases are passively absorbed and by dissociation, form ions in the plasma, which can be utilized in metabolic processes.

The mechanism of the active uptake processes is not known, neither is that of the introduction into the plasma nor that of the secretion by the plasma into the vacuole.

It may be, that the ion pairs are introduced into the plasma with the aid of free energy potentials supplied by energy-rich substances, it can also be presumed, that the ions combine with carriers, with either a carrier for each ion, or one carrier for an ion pair, as was suggested by STEWARD and STREET (1947). In this case cations and anions together with the carriers move as complexes, diffusing through the membrane. It is commonly assumed, that the complexes are lipid soluble and therefore penetrate the lipid plasma membranes. After having passed through the membrane the ion-carrier complexes are decomposed and cations and anions appear in the ion pool of the plasma compartment (Chapter I and IX D).

It was proved by experiment (ARISZ, 1953, 1958, VAN LOOKEREN CAMPAGNE, 1957) that the biochemical processes involved in active uptake use free energy potentials, supplied by energy-rich substances produced by respiration, photosynthesis or other processes in the cell.

BENNET CLARK (1956) has proposed a mechanism of carriers binding sugars, water and ion pairs at the boundary of the cytoplasm containing lecithin or lipoproteins. The complex is hydrolysed at the inner side of the membrane by lecithinase. Such complexes of carriers with cations and anions could play a role, particularly in the secretion of ions through the tonoplast into the vacuole.

The specific nature of the concurring cations and anions influences the rate of transport through the membrane. It has often been mentioned that the quantities of cations and anions, absorbed from a salt solution, are not equal.

The presence of excess ions has been interpreted as the result of a complementary exchange between ions in the plasma and ions in the external solution. Hydrogen ions in the plasma were assumed to exchange with potassium ions in the external solution and bicarbonate ions in the plasma produced during respiration, were assumed to exchange with anions in the external solution. (PIRSCHLE and MENGDEHL, 1931). Such exchanges of chloride ions, do not occur as long as the membranes are in their primary state and remain impermeable to chloride ions. (In regard of other ions cf. PIRSCHLE, 1932).

The problem of unequal cation and anion uptake is one of the most interesting problems of the biochemical side of uptake processes. It is intimately connected with metabolism, since it is accompanied by changes in the organic acid content of the leaves (ULRICH, 1942, BURSTRÖM, 1945).

From a solution of potassium chloride often as many cations as anions are absorbed, but it also happens, that more equivalents of chloride ions are absorbed than of calcium ions. In this case the organic acid content of the leaf is increased. In the uptake of potassium sulphate and phosphate more equivalent potassium usually is absorbed than sulphate and phosphate and at the same time the quantity of organic acid anions in the leaf is increased. The formation of organic acid anions seems to be just sufficient to balance the excess of potassium ions.

When calcium chloride is absorbed the amount of equivalent chloride ions absorbed is often larger than that of the equivalent calcium ions. In this case there is an excess uptake of anions and a decrease of organic acid anions.

HOAGLAND and DAVIS (1929) found, that in *Nitella* an excess uptake of cations was compensated by the formation of organic acid. Later observations of ULRICH (1942) and BURSTRÖM (1945) have made it clear that by the formation and the decrease of organic acids unequal cation and anion uptake is regulated.

I shall try to give a brief idea of the present state of this problem, in relation to the principles of active uptake expounded above. We start from the supposition of COLLANDER that the plasma membranes in their primary state are non permeable to ions like hydrogen, hydroxyl and bicarbonate, but that unionised molecules, having the power to permeate through lipid membranes, can pass the membranes in both directions. Molecules like carbon dioxide, ammonia and water penetrate the plasma from outside and from within and leave the tissue by diffusion. Arrived in the plasma, carbon dioxide and water dissociate into hydrogen and bicarbonate ions, ammonia and water in NH_4^+ and OH^- ions. Carbon dioxide is always present in living cells, where it is formed in the mitochondria by respiration.

If roots or discs of storage tissues or leaf segments of *Vallisneria* are placed in a bicarbonate solution, cations and anions are actively absorbed. If the bicarbonate is labelled with C 14 or C 11 it can be shown after uptake that labelled carbon is introduced into the tissue. This was found for roots by OVERSTREET, RUBEN and BROYER in 1940. The process was further studied by JACOBSON, (1955), JACOBSON and ORDIN (1954), HURD (1958, 1959), HURD and SUTCLIFFE (1957), HUFFAKER e.a. (1960), BAHN e.a. (1960), JACKSON e.a. (1959).

The bicarbonate ions cannot pass through the non-permeable plasma membrane. As there is a continuous production of carbon dioxide in the cells by respiration and the carbonic acid molecules dissociate in H^+ and HCO_3^- ions it can be expected that part of the labelled bicarbonate ions in the plasma will be interchanged for unlabelled bicarbonate ions. This has the effect, that only part of the labelled bicarbonate ions just actively introduced into the plasma from the external solution remain in the plasma and that labelled carbon dioxide molecules leave the tissue.

A second process occurs in the plasma, which transforms the remaining labelled bicarbonate ions and the unlabelled bicarbonate ions introduced by interchange, into organic acid anions by using the bicarbonate ions in a carboxylation process, which produces organic acid anions. In the secretion of ion pairs from the cytoplasm into the vacuole, cations are combined with an equal amount of anions, consisting in part of organic acid anions like malate, and partly of inorganic anions, which are available in the plasma. Consequently it can be expected, that the increase in the amount of organic acid anions in the tissue, equals the amount of bicarbonate anions absorbed from the external solution, but that the specific activity of

the organic acid anions is lower than that of the absorbed HCO_3 ions.

HURD stated, that the uptake of potassium and chloride from a potassium chloride solution may appear unequal and that more potassium than chloride is absorbed in a solution of high pH. He ascribes this inequality to the presence of bicarbonate ions in the external solution, which have also been absorbed. In this way the excess potassium absorption is only an apparent effect, because the uptake of bicarbonate ions was not taken into account.

It seems justified to say, that the active uptake of bicarbonate ions induces the plasmatic conversion of bicarbonate into malate ions. The statement of SUTCLIFFE, 1962: 51, that dark fixation of carbon dioxide in non-green tissues has an interesting effect on the differential absorption of anions and cations, confuses cause and effect. The uptake of the bicarbonate ions into the plasma is the primary cause of the formation of organic acid anions in the plasma.

The interpretation of the apparently unequal uptake of calcium and halogen ions in the absorption of calcium chloride and calcium bromide, which is accompanied by a simultaneous loss of organic acid anions seems to be rather more complicated.

In *Vallisneria* leaves ARISZ (unpublished results) found a larger irreversible equivalent uptake of chloride than of calcium ions in experiments on active uptake. ULRICH (1942) found, that from calcium bromide more equivalent bromide was absorbed than calcium but REDFERN (1922), investigating uptake from calcium chloride, mentioned a larger uptake of calcium than of chloride ions in *Pisum sativum* and *Zea Mays* and there are many more data of this kind in literature.

It is confusing, that in the older observations the uptake of cations by exchange has not been separated from the irreversible active uptake into the plasma. This makes interpretations dubious. The data of REDFERN can be explained by a supplementary uptake of bicarbonate ions. The larger equivalent uptake of chloride than of calcium ions, mentioned by ULRICH (1942) was accompanied with a simultaneous decrease of the organic acid anions in the tissue. It may be assumed, that next to an absorption of calcium and halogen ions in equal equivalent amount the excess anion uptake of chloride or bromide ions can be interpreted as absorption of HCl resp. HBr. The absorbed hydrogen and halogen ions come into the plasma, where organic acid anions are in store.

By combination of the excess hydrogen ions with organic acid anions organic acid is formed. In 1945 BURSTRÖM had already suggested that after the introduction of hydrogen ions into the cytoplasm the excess H ions combine with malate ions and disappear from the tissue since the concentration of malic acid is regulated by the Krebs cycle.

ULRICH (1942) showed by measuring the respiratory quotient, that the malic acid had been respired. In this way an apparent exchange of malate for chloride ions between the tissue and the external solution results. (Confer the discussion by STEWARD and SUTCLIFFE, 1959, by BRIGGS, HOPE and ROBERTSON, 1961 and by SUTCLIFFE, 1962, where ion exchange has not been excluded).

FIRMAN BEAR (1950) studying the mineral composition of alfalfa plants found that the milli equivalent sums of the cations K + Ca + Mg + Na and of the anions N + P + S + Cl + Si tended to be constant regardless of wide variations in the number of m.cq. of the individual cations and anions.

The cation-anion equivalent relationship at any given pH value could be expressed by $\dfrac{K + Ca + Mg + Na}{N + P + S + Cl + Si}$ = constant.

These cation-anion ratios were for the whole plant rather constant but varied for different parts of the plant.

Nitrogen in the plant is largely present in the form of proteins and amino acids. It is considered in the formula as nitrate. BEAR rightly remarks, that whatever is found for the cation-anion equivalent relationship electrostatic balance in the plant must be maintained. He assumes that when a nitrate ion is assimilated into protein an organic anion must arise or a hydrogen ion must disappear to compensate.

BEAR assumes that this cation-anion relationship is the consequence of the absorption of mineral salt ions and considers the increase in nitrogen as nitrate absorption. He mentions that uptake of nitrogen can also be effected as ammonium ion. In that case the absorption of other cations is reduced or that of anions P + S + Cl + Si is enhanced.

Since nitrogen at higher pH can also be absorbed as molecular ammonia the interpretation of the nitrogen uptake as nitrate absorption is rather arbitrary. This has no consequences in regard of the discreet use made by BEAR of his formula, but it is unacceptable when on the ground of such a balance conclusions are drawn about ion uptake.

DIJKSHOORN (1958) studying nitrate accumulation, nitrogen balance and cation-anion ratio during the regrowth of perennial Rye grass considers nitrogen increase after manuring with ammonium nitrate as nitrate absorption.

He assumes active uptake of nitrates and passive exchange of nitrate ions from the external medium for hydroxyl or bicarbonate ions. In my opinion the data of the ionic balance equation are unsufficient for a discussion of the nature of the uptake processes.

In a recent publication DE WIT, DIJKSHOORN and NOGGLE (1963) give an interesting survey of the litterature concerning uptake of cations and anions.

They consider their data insufficient to make speculations regarding the physiological mechanism involved in the regulation of the uptake. Their results agree with the concept of paired uptake if excess cation uptake is balanced by the uptake of OH or HCO_3 ions and excess anion uptake by the uptake of H ions. But they state that the difference between cation and anion uptake can also be balanced by exchange of bicarbonate or hydrogen ions. As ion exchange is at variance with the results of this publication only the first possibility remains.

Anions like nitrate must be absorbed together with a cation. If

the nitrate ion in the plasma is reduced to NH_3 the negative charge is released as HCO_3^- since carbondioxide is always present in the cytoplasm. As we have seen, bicarbonate ions in the cytoplasm are often used in a carboxylation reaction and organic acid anions are formed.

It is quite understandable that PIERCE and APPLEMAN (1943) determining the difference between the cation and inorganic anion content in different plant species found that the excess of cations is balanced by organic anions. This now can easily be understood on the basis of uptake of cations with bicarbonate ions.

F. *Influx of anions other than chloride*

Nearly all the experiments discussed in this publication refer to the uptake of chloride anions or to that of asparagine dipolar ions. Although many experiments have been carried out with other anions, they have not yet given a clear insight into the behaviour of these anions in plasmatic processes.

The multistep theory (ARISZ 1953, 1958, 1960b, 1963a) assumes that uptake consists of several consecutive steps. After the ion pairs have passed through the peripheral plasma membrane into the cytoplasm, the ions in the cytoplasm are involved in at least three different processes:

1. the migration of ions in the symplasm;
2. the secretion of ions from the cytoplasm into the vacuole;
3. metabolic processes in the cytoplasm.

The ratio between the amount of ions involved in each of these plasmatic processes, must be different for the various anions and it is to be expected that in different plants strongly divergent ratios will be found. The discussion here will be confined to data relating to *Vallisneria*.

Bicarbonate, phosphate, sulphate and nitrate ions are more involved in metabolic processes than chloride ions. Bicarbonate ions absorbed into the plasma seem to take part in the formation of organic acids; inorganic phosphates are used in the formation of organic phosphates, and nitrate and sulphate ions will be used in the synthesis of amino acids and proteins.

It cannot be said whether the secretion mechanism can secrete all different kinds of anions into the vacuole, because the vacuole contents cannot be separated from the ions of the plasma pool in *Vallisneria* cells; but, as nitrate uptake is mostly small and is increased by factors which promote cell metabolism, accumulation into the vacuole plays no important role here. Secretion of ions into the vacuole is an irreversible process, but the possibility cannot be excluded that ions can be returned from the vacuole into the plasma by another independent active process, however, no evidence for such a process is available. ARISZ (1954) found no diffusion of ions within the symplasm between vacuoles of adjoining cells, though a small leakage from the

vacuoles could not be excluded. Such leakage can be a secondary effect, as has been shown in this publication.

G.   *Influx of cations*

WINTER has studied the uptake of cations in *Vallisneria* leaves, particularly with regard to labelled rubidium and labelled calcium ions. His results were in agreement with BRIGGS and ROBERTSON, showing that the absorbed rubidium ions can be arranged in three fractions: a. ions in the free space, b. ions in the Donnan space, exchanging with identical cations in the external solution, c. irreversibly absorbed ions rarely in the wall mostly in plasma compartments.

The amount of rubidium ions in fraction b is in almost linear proportion to the concentration of rubidium in the external solution. Changes in the amount of rubidium ions in fraction b can be brought about in solutions with other cations, like hydrogen, calcium, potassium and sodium.

The same three fractions have been found for calcium ions. It is remarkable that even with low external concentrations of calcium the amount of calcium ions in fraction b is many times higher than the amount in the irreversibly absorbed fraction c.

An important problem is whether in the primary state the membrane is non-permeable to different cations. The large amount of cations in fraction b makes it impossible to use the methods of Chapter III for demonstrating leakage and exchange of cations in the plasma after active uptake.

WINTER (1961) has given some attention to the location of the exchangeable fraction b of labelled rubidium ions in *Vallisneria* cells. He wanted to know whether the capacity of the isolated cell wall to exchange rubidium ions, was as great as that of intact cells. In that case there is no fraction b in the cytoplasm. He measured the exchange capacity of the isolated cell wall by the adsorption of labelled lanthanum ions, because the adsorption of these trivalent ions is not influenced by interfering univalent and divalent cations. Practically the whole of the lanthanum fraction (about 96 %) was localized in the cell wall, and WINTER therefore assumes that the adsorbed rubidium ions are also located mostly in the cell wall. This result does not exclude the fact, that an insignificant amount of exchangeable rubidium ions is located in the plasma membrane, probably at its boundary with the medium. It seems, however, unlikely that cations situated inside the plasma membrane are able to exchange with ions in the external solution. As there are many potassium ions in the plasma pool, such an exchange cannot be overlooked.

It can be expected that these ions would have exchanged with rubidium ions in the external solution if the plasma membrane had been permeable, so it is unlikely, that in the primary state the peripheral plasma membrane is permeable to potassium and rubidium ions.

The improbability of an exchange of cations through the plasma membrane appears also from other considerations. If the chloride ions are actively absorbed from a potassium chloride solution and the amount of the irreversibly absorbed potassium ions is as large as that of the chloride ions, it is clear that only ion pairs of potassium and chloride have been absorbed. Often in *Vallisneria* as well as in other materials an equal uptake of cations and anions has been stated and because it has been proved, that the uptake of chloride ions is not the consequence of exchange but is an active uptake process the uptake of cations to the same amount as of anions can only be the consequence of the uptake of ion pairs. The equal amounts of cations and anions indicates, that the membrane impedes cation exchange. An unequal cation and anion uptake has to be interpreted in the way discussed in E.

Another way to study the exchangeability of cations in the plasma with identical ones in the external solution has been explored by ARISZ (1960a). He studied exchange of labelled rubidium ions which were introduced into the symplasm in the 2.5 cm absorbing part of a 7.5 cm leaf length, while they were migrating in the symplasm of the adjoining part of the leaf length.

This part was surrounded by a potassium chloride solution without labelled rubidium ions, which gave migrating labelled rubidium ions the opportunity to exchange with potassium ions in the external solution. In this experiment, only in the absorbing part of the leaf length were labelled rubidium ions adsorbed in the walls in fraction b. It may be expected, that in the adjoining zone the migrating labelled rubidium ions were exchanged with the potassium ions of the external solution, if the plasma membranes allow them to pass through. Up to now exchange has not been found, which indicates that labelled rubidium ions and potassium ions are separated by a non permeable peripheral plasma membrane. A final result, however, has not yet been obtained. These translocation experiments will be continued and discussed in a following publication. The available evidence indicates that material in the primary state does not exchange cations through the plasma membrane. In the secondary state, when the membrane has increased its permeability, leakage takes place.

H. *Influx and efflux with tissues other than Vallisneria leaves*

The influx-efflux scheme of BRIGGS and ROBERTSON has focussed attention on the necessity of determining influx as well as efflux for all different tissues commonly used in uptake experiments and moreover the necessity for finding out the relation of these fluxes to metabolism and external and internal factors.

Active and passive processes of ion uptake do not exclude each other; their relative importance may differ for anions and cations and it seems likely that the behaviour of different ions depends on their specific features.

Resistant *Vallisneria* leafcells have plasma membranes which are

impermeable to ions, but changes brought about by a slight alteration in the external solution or by an injury to the plasma increase their permeability to ions, so that exosmosis results. In such systems, a combination of influx and efflux is to be expected.

Exosmosis is only a temporary phenomenon; impermeability of the membrane seems to be restored either naturally after a period of time, or under the influence of external factors such as a supply of light, or calcium ions, or by removal of injurious substances from the external solution, or by factors influencing metabolism and the supply of energy. Formulae which are not based on sufficient physiological knowledge of the processes involved have been used for describing the time course of exosmosis. The dimensions of the compartment from which exosmosis occurs are mostly unknown. It may be the "plasma compartment" alone or in combination with other compartments especially the vacuole. When the permeability of plasma membrane and tonoplast change independently by injury and recovery, the physiological conditions are so complicated that simple formulae cannot be used, because much simplification of the real conditions is required.

There is no doubt that flux analysis is necessary in all materials and that this will provide a valuable basis for comparative physiological and ecological purposes. The evidence from work with *Vallisneria* leaves makes it clear that in future more attention will have to be paid to the physiological foundation of the fluxes and rather big differences may occur in different plants. *Vallisneria* as a water plant has exceptional advantages as transpiration does not influence uptake and translocation. It seems that the roots of many plants are also rather sensitive to manipulation and a change in permeability of the membrane is readily brought about. Discs of storage organs are ideal material for many purposes, but since wounds have to be made by cutting thin discs, their reaction will depend on their resistance to wounding and their power of recovery. Some of these tissues are rather resistant, others seem to lack resistance and the increased permeability of the plasma-membrane continues for a longer period.

Much attention has been paid to the large cells of the *Characease* and to coenocytes like *Valonia* and *Halicystis*. OSTERHOUT assumed that the *Valonia* protoplast is not permeable to ions. STEWARD and MARTIN found that cells of *Valonia macrophysa* brought into the laboratory leaked as a result of mechanical injury. In this respect they react in the same way as *Vallisneria* cells. Much information about uptake on the *Characeae* has been gathered in the course of time, for example MAC ROBBIE and DAINTY have studied the fluxes in these plants during the last few years. As it is not my intention to discuss the results obtained from experiments on fluxes but only to consider the physiological systems involved, I will confine the discussion to the researches of COLLANDER and his coworkers on the uptake of salt ions by members of the *Characease*.

COLLANDER has given some interesting data on the difficulties encountered in obtaining good results from ion uptake experiments.

It seems that these difficulties are to a large extent caused by the toxicity of the ions used, and the changes in permeability inflicted upon the membranes of the individual cells during the experimental period. Some older observations by COLLANDER on the uptake of cations are of particular interest since they are in close agreement with the results obtained with *Vallisneria* leaves.

COLLANDER and his coworkers have investigated the permeability to both electrolytes and non-electrolytes. COLLANDER and BÄRLUND have shown that with isolated cells accurate and reliable determinations of permeability to organic non-electrolytes can be obtained. But his experiments with members of the *Characeae* on the uptake of cations did not give such good results (1939). COLLANDER used internodal cells of the "leaves" of *Chara ceratophylla* and internodal cells of "stems and leaves" of *Tolypellopsis stelligera*. In particular, he studied the uptake of lithium ions since these could easily be measured by quantitative spectral analysis. In spite of the accurate methods he used, he did not succeed in getting a clear separation of the active and passive uptake of lithium ions. The lithium absorbed was not given off into the water during a period of two days and he came to the result "that the absorption of cations by the cells of *Chara* and *Tolypellopsis* underlies the same general rules as the absorption of anions by another Characean plant, viz *Nitella clavata* studied by HOAGLAND. In many respects, however, the results of the present study are incomplete." The difficulties can be partly understood in the light of more recent experiments. We would mention the following points:

1. It is not unexpected that experiments regarding lipid permeability present fewer difficulties than those regarding active uptake of ions. This seems to be connected with the different pathways used (HÖFLER). Lipophilic substances follow the lipid pathway and ions follow the pore pathway. The former can be restored perfectly whilst the pore pathway is easily altered by different factors. Restoration is possible but it is a slower process.
2. The lithium ion is not an ion needed for normal growth, and is commonly considered as toxic, this effect being more pronounced when uptake is increased, as in the light.
3. As cations lithium ions are adsorbed on the wall and bound in fractions b and c; COLLANDER did not distinguish between these fractions. The amount of lithium ions in fraction b seems to be considerable. Exosmosis of actively absorbed ions can be expected as a result of leakage by the plasma membrane.
4. COLLANDER observed that several cells, after having been isolated from their adjoining cells, lost their turgidity. Such cells and also those which did not show protoplasmic streaming were discarded at the end of the experiment. The remaining cells were considered to be healthy and capable of giving reliable results. These criteria are not quite satisfactory, since there are many gradations between cells which have lost their turgidity and those with unchanged permeability.

Also the presence of protoplasmic streaming is no guarantee of a healthy condition, because, according to observations of JAGER (1958) toxic cations like sodium and lithium induce protoplasmic streaming in darkened *Vallisneria* leaves, while in presence of potassium and calcium ions the darkened protoplasm need not exhibit streaming.

In these experiments COLLANDER used a set of cells for each determination and obtained average values from a mixture of cells with different degree of leakage. Reliable results for non-leaking cells cannot be obtained in this way. In 1954 COLLANDER altered his method and mentioned, that each cell was kept separate and that uptake was measured by analysing the external solution at different times.

In material which shows variable results this is the appropriate way of determining the uptake of one distinct cell under various conditions.

Before the cells can be used in the experiments they have to be isolated from their adjacent cells. COLLANDER mentions that in isolating the cells a considerable degree of mechanical irritation cannot be avoided. In 1954 he studied the suitability of cells of *Nitella mucronata* for these experiments. "It was not thought possible to remove the small nodal cells from the much greater internodal cells but owing to their smallness they cannot have affected the results to any considerable extent". Thinking of the symplasmatic connections between the internodal cells and the adjoining small nodal cells I am not so sure that the small dimensions of these cells give any guarantee against leakage along their symplasmatic tracks. I am not convinced that the cells used in these experiments had completely returned to their primary condition before being isolated.

COLLANDER carried out a useful experiment by cultivating young plants of *Tolypellopsis* in a nutrient solution for three months. The plants behaved quite differently from the older isolated cells. They accumulated potassium and rubidium to a level 25 to 30 times greater than the concentration of the external solution, whereas lithium ions and sodium ions were accumulated to a concentration which was only 1–4 times greater.

A *Chara* plant is fixed in the soil by means of rhizoids and presumably substances are absorbed from the soil (BIERBERG 1909). These substances have to be translocated in the plant from cell to cell along the symplasm in the same way as translocation has been observed in the leaves of another waterplant, *Vallisneria*. In such a system, continuous leakage from the symplasm would be most unlikely, as COLLANDER explained in 1939, because the result of leakage is a waste of energy. It seems attractive to consider the possibility that healthily growing *Chara* cells just like *Vallisneria* leaf cells, have plasma membranes, which are non-permeable to electrolytes.

I. *Infiltration of the tissue as general indicator of locally increased membrane permeability*

Tissue infiltration can be considered as an indicator of the labile state of the plasma membrane, which leads to increased permeability and loss of solutes from the cells. The intercellular spaces become filled with this solution.

When *Vallisneria* plants are cultivated in large concrete tanks it has been observed that the day after the leaves have been thoroughly cleaned, a local infiltration of the leaves occurs. As a result of the manipulations local changes in the membrane permeability arise, which cause an infiltration of the tissue. The process is generally irreversible and leads to local injury, which is progressive. If vigorously growing resistant plants have not been irritated in any way, they do not show infiltration or increase in permeability. Leaves showing signs of infiltration should not be used for uptake experiments.

Infiltration is a general phenomenon which is not confined to *Vallisneria* leaves, it is also found in roots (J. M. JANSE, 1926). The phenomenon was studied in detail by Miss M. F. E. NICOLAI (1929) in roots of the *Cruciferae* and *Helianthus annuus*. The effect can be obtained in various ways e.g. by transferring the roots from the soil into water, by cautiously rubbing or pencilling them, or by putting them into salt solutions. It can also be observed after a sudden increase of temperature to 35 C.

Infiltration indicates the extreme sensitivity of the root system to manipulation and to change in the external solution, which, in effect, means the use of unbalanced solutions. Apparently the phenomena of infiltration in roots and in *Vallisneria* leaves are similar. Analogous reactions are frequently obtained by the stimulation of tissue cells e.g. by seismonastic stimuli and by pathological changes in membrane permeability e.g. in the shoot tissue of tulips. (PINKHOF 1929).

SUMMARY

In this publication the classical view that the plasma membranes are able to exclude the passage of electrolytes, has been corroborated. These substances must be introduced into the cellplasma by the expenditure of free energy potentials.

The structure of the peripheral plasma membrane, which in its primary state is impermeable to chloride ions can be changed by several external and internal factors so that its permeability to electrolytes is increased. As a result, leakage of the cell contents occurs to varying extents. The change of the membrane permeability depends on what may be called the grade of resistance of the tissue. Moderately resistant material has been used for the present research. During uptake the impermeability of the peripheral plasma membrane is usually maintained, but leakage often occurs subsequently.

A method developed in Part I (Protoplasma 57, 1963) has been used for assessing the permeability of the membrane to chloride ions. The method and the results obtained are described fully in Chapter III.

Secondary changes in permeability can be the result of changes in membrane structure caused by cutting the leaves (Chapter IV and V). Changing the ionic composition of the external solution either improves or diminishes the increase of permeability. This is the result of the antagonistic influence of ions, particularly potassium and calcium, on the membrane permeability. A solution in which the

non-permeability of the membrane is maintained has been called a balanced solution. The balanced solution of OSTERHOUT, STILES, HOMÈS and others is essentially a solution which maintains the non- permeability of the plasma membrane to electrolytes.

In the secondary state of enhanced permeability, influx and efflux occur side by side. If the conditions both inside and outside are favourable, the increased permeability of the membrane is of limited duration. Sooner or later a recovery sets in, which restores the primary state of impermeability of the membrane to electrolytes.

The uptake of asparagine is also an active process, and displays the same features as salt uptake. The peripheral membrane is impermeable to the asparagine ions, presumably dipolar ions, but in secondary state the permeability is increased. The antagonistic effect of potassium and calcium ions is remarkable but by using balanced solutions the impermeability of the peripheral membrane generally remains unchanged.

The consequences of the experimental results have been outlined in the discussion (Chapter 9). Arguments have been put forward to suggest that the non-permeability of the plasma membrane is maintained for cations and for anions other than chloride.

Consequently it appears that the uptake of salts into the cytoplasm is not the result of exchange processes, but is the result of the active absorption of ion pairs. It has been explained how an apparently unequal cation and anion uptake can be realized as a result of the simultaneous uptake of combinations of other cations and anions containing either hydrogen or bicarbonate.

In effect, therefore, it is preferable not to speak of the permeability of the membrane to ions but to ion pairs. Finally there has been a discussion of whether a primary state of non-permeability of the plasma membrane and a secondary increase of the permeability by external and internal factors can also be found in other tissues than *Vallisneria* leaves, such as storage organs, large cells of the *Characeae* and roots.

There is a considerable quantity of data which points to the uniformity of the behaviour of these different cell systems. The differences found have to be interpreted as variations of the general scheme of influx and efflux designed by BRIGGS, HOPE and ROBERTSON.

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