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Kinetics of Nonionic Diffusion of Hydrogen Fluoride in Plants
I. Experimental and theoretical treatment of weak acid permeation[1])

By

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With 6 Figures

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Summary

KRONBERGER W. 1987. Kinetics of nonionic diffusion of hydrogen fluoride in plants, I. Experimental and theoretical treatment of weak acid permeation. – Phyton (Austria) 27 (2): 241–265, with 6 figures. – English with German summary.

Uptake of F into single plant cells *(Saccharomyces cerevisiae)* and tissues (turions of *Spirodela polyrrhiza,* leaf or needle slices of *Fagus sylvatica* and *Picea abies*) was investigated. Results indicate a passive permeation of the undissociated HF molecule across the plasmalemma. Thus HF behaves like other weak acids, where the ionic species becomes trapped within membrane surrounded compartments after nonionic diffusion.

Kinetics of F uptake from a small, definite volume of an outer medium was followed continuously until equilibrium. A theory was derived describing the kinetics of weak acid permeation. Experimental data fit to the theory indicating that HF permeation obeys first order kinetics. The permeability coefficient for HF depends on the pH of the medium and ranges from 0.87 to 2.9 10^{-7} ms^{-1} (at pH 3.1 to pH 6, 25° C) in *Spirodela* turions. In yeast cells permeability is probably of the same order of magnitude, however, uptake deviates due to binding of F. Permeability for HF is about 10 times lower than permeability for H_2O. This relationship is most easily predicted from the hexadecane:water partition coefficients.

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[1]) Dedicated to Professor Dr. Riklef KANDELER on the occasion of his 60th birthday.

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Zusammenfassung

KRONBERGER W. 1987. Die Kinetik der Diffusion von undissoziiertem Fluorwasserstoff in Pflanzen, I. Experimentelle und theoretische Behandlung der Permeation einer schwachen Säure. – Phyton (Austria) 27 (2): 241–265, mit 6 Figuren. – Englisch mit deutscher Zusammenfassung.

Die Fluor-Aufnahme in einzelne Zellen *(Saccharomyces cerevisiae)* und in Gewebe (Turionen von *Spirodela polyrrhiza,* Blatt- oder Nadelschnitte von *Fagus sylvatica* und *Picea abies*) wurde untersucht. Die Ergebnisse zeigen, daß HF Moleküle passiv über das Plasmalemma permeieren. HF verhält sich also wie andere schwache Säuren: nach Permeation der undissoziierten Species und deren Dissoziation werden die Ionen in einem Kompartiment gefangen (Ionenfalle).

Die Kinetik der Fluor-Aufnahme aus einem kleinen, definierten Volumen eines äußeren Mediums wurde kontinuierlich bis zur Gleichgewichtseinstellung gemessen. Eine Theorie zur Beschreibung der Kinetik der Permeation schwacher Säuren wurde abgeleitet. Die experimentellen Daten stimmen mit der Theorie überein und zeigen, daß die Permeation von HF einer Kinetik erster Ordnung gehorcht.

Der Permeabilitätskoeffizient für HF ist vom pH des Mediums abhängig und reicht bei den Turionen von *Spirodela* von 0.87 bis 2.9 10^{-7} ms^{-1} (bei pH 3.1 bis pH 6, 25° C). Bei Hefezellen weicht die Aufnahmekinetik infolge der Bindung von F in den Zellen ab, doch liegt der Permeabilitätskoeffizient in der gleichen Größenordnung wie bei den Turionen. Die Permeabilität für HF ist ungefähr 10 mal geringer als die für H_2O. Dieses Verhältnis wird am besten durch die Hexadekan:Wasser – Verteilungskoeffizienten dargestellt.

Introduction

Toxic effects of inorganic fluoride compounds on biological systems are widely investigated. Plants are among the most sensitive organisms, particularly when contaminated with atmospheric HF from air pollution (N.A.S. 1971, WEINSTEIN 1977, DAVISON 1982). To understand toxicity of fluoride compounds, it is necessary to have knowledge also of their mode of entry into, distribution within, and loss from an organism. There still exists lack of information on fluoride uptake into plants, especially in which way fluorides cross plasma membranes. Is it the fluoride ion which crosses membranes passively? It is known that ions can pass a membrane passively only to a very small degree. Or is F^- taken up actively by means of a carrier? This seems quite unlikely for an element having no essential function in plants, not even that of a trace element. F^- rather could be mistaken for a chemically related ion, e. g. Cl^-, in a similar way as Rb^+ is taken up instead of K^+.

Beyond that, as F^- is the ionic form of a weak acid, it could permeate in its nonionic form as the HF molecule. The first suggestion of a nonionic diffusion of HF into the living tissue originates with WIELAND & KURTZAHN (1923) from the following observation: a small droplet of hydrofluoric acid, brought on the skin of the forearm, very soon causes a reddish swelling beneath the intact epidermis; on the other hand a droplet of hydrochloric

acid dries up without any symptoms of injury. The authors concluded that the highly ionized hydrochloric acid is not able to penetrate the living tissue, whereas the weak HF in its nonionic form is. There now exists a great deal of experimental evidence for HF being the species permeating through biological membranes in a great variety of organisms, such as bacteria (ROBERTS & RAHN 1946, SHIOTA 1956, EISENBERG & MARQUIS 1980, SCHUSTER & al. 1981), yeast cells (RUNNSTRÖM & SPERBER 1938, MALM 1940, 1947, ROBERTS & RAHN 1946, SIMON & BEEVERS 1952), animals (WHITFORD & al. 1977, REYNOLDS & al. 1978, WHITFORD 1983), and humans (JÄRNBERG & al. 1981, EKSTRAND & al. 1982). Furthermore, GUTKNECHT & WALTER (1981) studied permeabilities of a planar lipid bilayer membrane to HF and F^-. They found a permeability for HF more than six orders of magnitude higher than for F^-, which supports the concept of nonionic diffusion also for natural membranes. However, for autotrophic plant cells there exists a surprising lack of interest in this topic. With the exception of EDDINS (1959) and MCNULTY & LORDS (1960), who found some evidence for HF permeation into cells of *Phaseolus vulgaris* (bush beans) and *Chlorella pyrenoidosa* respectively, no experimental treatment has been reported in the literature. MILLER & al. (1986), in a predictive model, discussed subcellular F distribution after diffusion equilibration of HF. They stated that this mechanism could explain the inconsistency of whole – plant F concentration with the apparently higher *in vitro* concentrations necessary for enzyme inhibition.

The objective of this work was to investigate whether nonionic diffusion of HF is a predominant mechanism of F uptake into plants. Furthermore, a theoretical background is established to evaluate kinetics of weak electrolytes for both experimental and natural situations.

Material and Methods

a) Plant material

Spirodela polyrrhiza, strain P 143, was grown aseptically in cotton-stoppered 300 ml Erlenmayer flasks on 200 ml autoclaved nutrient solution. Medium 4 after DATKO & al. (1980) was used (modified by replacing NH^+_4 by Ca^{++} and K^+). Plants were irradiated (OSRAM HQIL lamps) with 60 $\mu E\ m^{-2}\ s^{-1}$ (determined with a LI-COR 188 quantum radiometer) for 16 hours per day. Temperature was 26° C during the light phase and 22° C in the dark. Turion formation was induced either when plants became overcrowded and nutrients partly exhausted (FÄRBER & al., 1986), or by adding abscisic acid (10^{-7} mol l^{-1} nutrient solution) according to SMART & TREWAVAS (1983 a). After turions had settled they were separated from the nutrient solution, washed from root debris, transferred to fresh nutrient solution, and stored at 4° C until use. Before each uptake experiment, turions were suspended in a F-free incubation medium (0.05 molar citric acid – KOH buffer in half

strength nutrient solution) for pH, thermal and osmotic equilibration. Unless otherwise stated, this preincubation was performed for two hours. After that, the suspensions were poured on a suction funnel to get a homogeneous layer of turions. Suction always was continued for 2 minutes to remove adherent water as uniformly as possible. From the turion "pellet" obtained in this way, weighed amounts were taken for measurements of F uptake, extraplasmatic spaces, density, and dry weight.

Commercial bakers' yeast (*Saccharomyces cerevisiae,* MAUTNER-MARKHOF AG, Wien) was suspended in tap water, centrifuged and resuspended twice in a 0.1 molar incubation medium (0.1 molar citric acid – KOH buffer in half strength nutrient solution). The last centrifugation always was performed after two hours of preincubation at 4000 *g* for 5 minutes to get a standardized pellet, from which the yeast cells were taken for measurements.

Leaf and needle slices were prepared from field grown beech (*Fagus sylvatica* L.) and spruce (*Picea abies* [L.] KARST.) trees. Beech leaves were cut crosswise with a set of parallel razor blades to 1 mm^2 squares, while avoiding the region of the mid rib. Spruce needles (current year's growth) were cross-sectioned in pieces of 1 mm length. Tissue slices were washed twice with distilled water and with the incubation medium on a suction funnel. For each experiment they were prepared freshly.

b) F uptake experiments.

Suspensions of turions, yeast cells or tissue slices were prepared with small volumes of incubation medium, and the decrease of F in the medium due to uptake into the cells was followed continuously. Measurements were performed at 25 ± 0.1° C in a thermostated water bath. Suspensions were stirred magnetically throughout in polyethylene bakers. A fluoride selective electrode supplemented with a single junction reference electrode (No. 94–09A, 90–01, ORION Research Inc.) and a pH electrode (No. LoT 406–M3, INGOLD) were properly positioned in the suspension. The mV-readings of the electrometers (Model 701, ORION) were traced on strip chart recorders (Servogor RE 646, GOERZ) as a function of time. To start an experiment, a certain amount of KF was added by means of a microsyringe to yield the desired initial F concentration.

The F^- selective electrode was calibrated in terms of F concentrations (c^F). Calibration standards were made from the incubation medium used for each individual experiment by adding known amounts of KF. From the measured c^F and pH values in the suspension, HF and F^- concentrations (c^{HF}, c^{F^-}) were calculated as $c^{HF} = c^F/(1 + 10^{pH-pK})$ and $c^{F^-} = c^F/(1 + 10^{pK-pH})$, both derived from the HENDERSON-HASSELBALCH equation: $pH = pK + \log (c^{A^-}/c^{HA})$. The pK value of 3.1 for HF (at 25° C in a 0.05–0.1 molar ionic

background) is taken for calculation (BOND & HEFTER 1980: summary of "best" values). This value is assumed to be also valid for the interior of cells.

The pH electrode was calibrated at 25 ± 0.1° C with 0.1 molar phosphate and citrate buffers. F content of plant material was analysed after LEVAGGI & al. (1971). Evaluation of data was performed on a programmable calculator (TI–59, TEXAS INSTRUMENTS).

c) Determination of cell dimensions.

Volume densities of the turion and yeast cell pellets were measured pycnometrically. Extraplasmatic volumes were determined with the aid of radiochemicals; a weighed amount of the pellet was suspended in a small volume of the incubation medium containing ^{3}H-dextran (molecular weight 70 000) or ^{14}C-sorbitol and equilibrated at 25° C. Dilution of the labelled compounds in the supernatant was measured by counting in a liquid scintillation spectrometer (LS 330, BECKMAN; scintillation cocktail: Ready Solve-MP, BECKMAN). From the dilution of ^{3}H-dextran the volume of adherent water and from dilution of ^{14}C-sorbitol the volumes of adherent water plus cell wall free space were calculated. To determine intracellular volume and plasmalemma surface area of the turion pellet, data from a stereological analysis performed by SMART & TREWAVAS (1983 b) on *Spirodela polyrrhiza* turions were taken together with the data for the extraplasmatic spaces. The dimensions of the yeast cells (the larger and smaller protoplast diameter of each of 872 cells, suspended in the incubation medium) were measured in a light microscope (REICHERT, Zetopan). Data for volume and surface area were calculated according to a procedure given by MUSFELD (1942) for ellipsoidic cells.

d) Determination of partition coefficients.

Hydrocarbon:water partition coefficients (K_{hc}), defined as activity of a solute in the organic phase divided by the activity of the solute in the water phase at equilibrium, were determined for HF, and as possible for F^- and the NaF ion pair, in various organic solvents. Polyethylene vials (50 ml) were rinsed with distilled water and the organic solvent concerned. 25 ml aqueous solution containing either HF or NaF, adjusted to an appropriate pH by addition of small amounts of HCl or NaOH, were layered with 25 ml of the organic phase. Vials were kept at 25 ± 0.1° C for 6 hours and shaken repeatedly. After centrifugation at 1000 *g* for 10 minutes, aliquots of both phases were withdrawn and analysed. F of the aqueous phase was analysed directly with the F^- selective electrode after mixing 1 : 1 with a total ionic strength adjustment buffer (TISAB II, ORION). F of the organic phase was measured after back-distribution into a small volume of 1 mM NaOH and after mixing the NaOH solution 1 : 1 with TISAB II. K_{hc} values of HF were determined in a pH range of 1.6 to 3.1 in the water phase; K_{hc} values of NaF

ion pair, F^- or ($NaF + F^-$) at pH 8. For calculation the ion pair dissociation constant for NaF in water of 1.88 (molal scale), the mean ionic activity coefficients according to ROBINSON & al. (1971), as well as the dissociation constant for NaF in n-octanol of 1.10^{-6} (measured by conductometry) were used.

e) Chemicals.

Chemicals used were obtained from MERCK, Darmstadt, in p.a. quality. Radiochemicals were purchased from AMERSHASM International. Olive oil was obtained from a local market. Glass-distilled water was used for preparation of solutions.

f) List of symbols used

S = indication of a nonelectrolyte,
also: amount of S (mol),
HA = indication of a weak acid, associated form,
also: amount of HA (mol),
A^- = indication of a weak acid, dissociated form,
also: amount of A^-,
HF = hydrogen fluoride, associated form,
also: amount of HF (mol),
F^- = fluoride ion, dissociated form,
also: amount of F^- (mol),
F = fluorine, sum of HF and F^-,
also: amount of F,
c = concentration (mol m^{-3}),
$\triangle c$ = concentration difference, driving force (mol m^{-3}),
V = volume (m^3),
t = time (s),
$1/\tau$ = time constant (s^{-1}),
$t_{0.5}$ = equilibrium half time (s),
A = area (m^2),
P = permeability coefficient (m s^{-1}),
R = resistance (s m^{-1}),
K = partition coefficient,
D = diffusion coefficient (m^2 s^{-1}),
$\triangle d$ = thickness of a barrier (m),
V_0, $V_{0-5\ min}$ = initial uptake rates ($\mu g\ g^{-1} h^{-1}$).

Subscript i refers to inside of a volume specified,
subscript o refers to outside of a volume specified,
subscript (t) refers to time from onset of permeation,
subscript equ refers to equilibrium of a permeator,

subscript hc refers to hydrocarbon phase,
subscript hd refers to hexadecane phase,
subscript cw refers to cell wall,
subscript pm refers to plasmalemma.

superscript tu refers to turion.

Symbols of substances are used also as superscripts.

Theory

Consider a system which contains cells (or a tissue) with a definite volume (V_i) inside the plasmalemma and a definite volume of an outer solution (V_o) to which at zero time ($t = 0$) a certain amount of a permeable nonelectrolytic solute is added. During the course of permeation the outer solute concentration (c_o^S) decreases, while the solute concentration inside the cells (c_i^S) increases. Permeation achieves equilibrium when $c_o^S = c_i^S = c_{equ}^S$, i. e. when the driving force ($\Delta c = c_o^S - c_i^S$) becomes zero. For simplicity, concentrations are considered rather than activities.

If it is possible to monitor c_o^S, one can determine c_i^S at any time (t), as the total amount of solute (S) in the system is given by

$$S = V_o\, c_{o(t)} + V_i\, c_{i(t)} \tag{1}$$

from which the amount of solute inside the cells (S_i) is determined by the second term of equation (1). The rate of permeation (dS_i/dt), based on Fick's First Law of Diffusion, is expressed by

$$\frac{dS_i}{dt} = A\, P\, \Delta c_{(t)} \tag{2}$$

where A is the surface area of the cells (plasmalemma area) and P is a measure of permeance (the permeability coefficient) or the reciprocal of resistance. Substitution of S_i and Δc, both derived from equ. (1), into equ. (2), and integration provide:

$$c_{i(t)}^S = c_{equ}^S \left[1 - e^{-\,t\,A\,P\left(\frac{1}{V_o} + \frac{1}{V_i}\right)} \right] \tag{3}$$

or in the general form: $c_{i(t)}^S = c_{equ}^S\,(1 - e^{-\,t/\tau})$, (4)

where the time constant $\frac{1}{\tau} = A\, P \left(\frac{1}{V_o} + \frac{1}{V_i} \right)$. (5)

From equ. (3) P can be calculated when $c_{i(t)}^S$ and c_{equ}^S are determined experimentally, and A, V_i and V_o are known. Alternatively, a plot of

$\ln[c^S_{equ}/(c^S_{equ} - c^S_{i(t)})]$ versus t should give a straight line with a slope of $1/\tau$, from which P can be determined according to equ. (5).

In a next step let us add an electrolytic solute, for example a weak acid, the associated form HA being permeable, while the ionic form A^- does not permeate. The concentration of the permeating species HA in the outer solution c_o^{HA} and that of the ion $c_o^{A^-}$ is governed by both the pH of the solution (pH_o) and the dissociation constant (pK_o) of the solute added. Reaching the interior of the cells, HA dissociates according to pH_i and pK_i. In a first approximation pH_i may be seen as an overall pH irrespective of compartmentation. The driving force ($\Delta c = c_o^{HA} - c_i^{HA}$) therefore depends strongly on pH and pK values. However, also in this case permeation achieves equilibrium when Δc becomes zero, independently of $c_o^{A^-}$ or $c_i^{A^-}$.

Thus, it can happen that at equilibrium $c_i^{A^-}$ is far higher than $c_o^{A^-}$ and that ions accumulate inside. This process, known as "ion trap mechanism" (see KINZEL, 1955) is caused by the pH difference across the membrane, the accumulating side being more alkaline in the case of a weak acid (or more acid in the case of a weak base) compared with the donor side.

The total amount of solute in a system containing an electrolyte is given by:

$$HA + A^- = V_o\,(c_o^{HA} + c_o^{A^-})_{(t)} + V_i\,(c_i^{HA} + c_i^{A^-})_{(t)} \qquad (6)$$

Applying the HENDERSON – HASSELBALCH equation, c^{A^-} can be expressed in terms of c^{HA}:

$$c_o^{A^-} = c_o^{HA}\,10^{(pH_o - pK_o)} \qquad (7a)$$

$$c_i^{A^-} = c_i^{HA}\,10^{(pH_i - pK_i)} \qquad (7b)$$

Equ. (6) can now be rewritten and simplified by introducing x_o for $1 + 10^{(pH_o - pK_o)}$ and x_i for $1 + 10^{(pH_i - pK_i)}$ to

$$HA + A^- = V_o\,c_o^{HA}\,x_{o(t)} + V_i\,c_i^{HA}\,x_{i(t)} \qquad (8)$$

The second term represents the amount of solute inside the cells ($HA_i + A_i^-$) at time t. The driving force, Δc, expressed in terms of c_i^{HA} may be derived from equ. (8) as follows

$$\Delta c = \frac{(HA + A^-) - V_i\,c_i^{HA}\,x_i}{V_o\,x_o} - c_i^{HA} \qquad (9)$$

Table 1.

Equations of uptake kinetics for nonelectrolytes and weak acids from a very large or from a small, definite volume of an outer medium. For explanation see text.

	NONELECTROLYTE, S		ELECTROLYTE (weak acid), HA	
	very large V_o	definite V_o	very large V_o	definite V_o
$c_{i(t)}$	$c^S_{i(t)} = c^S_o (1 - e^{-t/\tau})$	$c^S_{i(t)} = c^S_{equ} (1 - e^{-t/\tau})$	$c^{HA}_{i(t)} = c^{HA}_o (1 - e^{-t/\tau})$	$c^{HA}_{i(t)} = c^{HA}_{equ} (1 - e^{-t/\tau})$
$1/\tau$	$= A P \frac{1}{V_i}$	$= A P \left(\frac{1}{V_i} + \frac{1}{V_o}\right)$	$= A P \frac{1}{V_i x_i}$	$= A P \left(\frac{1}{V_i x_i} + \frac{1}{V_o x_o}\right)$
$t_{(0.5)}$	$= \frac{V_i}{A P} \ln 2$	$= \frac{V_i V_o}{A P (V_i + V_o)} \ln 2$	$= \frac{V_i x_i}{A P} \ln 2$	$= \frac{V_i x_i V_o x_o}{A P (V_i x_i + V_o x_o)} \ln 2$
P	$= \frac{V_i}{A t} \ln \frac{c^S_o}{c^S_o - c^S_{i(t)}}$	$= \frac{V_i V_o}{A t (V_i + V_o)} \ln \frac{c^S_{equ}}{c^S_{equ} - c^S_{i(t)}}$	$= \frac{V_i x_i}{A t} \ln \frac{c^{HA}_o}{c^{HA}_o - c^{HA}_{i(t)}}$	$= \frac{V_i x_i V_o x_o}{A t (V_i x_i + V_o x_o)} \ln \frac{c^{HA}_{equ}}{c^{HA}_{equ} - c^{HA}_{i(t)}}$

where $x_i = 1 + 10^{pH_i - pK_i}$ and $x_o = 1 + 10^{pH_o - pK_o}$

Substitution of $(HA_i + A_i^-)_{(t)}$ from equ. (8), and of Δc according to equ. (9) into equ. (2) yields after integration the general form of equation (4). However, contrary to a nonelectrolyte, $1/\tau$ for an electrolyte is now given by:

$$\frac{1}{\tau} = A\,P\left(\frac{1}{V_o\,x_o} + \frac{1}{V_i\,x_i}\right) \tag{10}$$

With the aid of equ. (4) and equ. (10), P of the nonionic form of an electrolyte can be obtained either by calculation or by plotting $\ln [c^{HA}_{equ}/(c^{HA}_{equ} - c^{HA}_{i\,(t)})]$ versus t. Details are given below. In an experimental setup, where V_o is chosen very large as compared to V_i, the outer solute concentration may be considered constant during permeation. It should be noted that for both a nonelectrolyte and an electrolyte the kinetics of permeation is represented by the general form of equation (4). Formally c^{HA}_{equ} has to be exchanged for c^{HA}_o (both of which coincide here). $1/\tau$ equals AP/V_i (ADAM, & al. 1977), and AP/V_ix_i (derivation not shown) for a nonelectrolyte and an electrolyte, respectively.

The four different cases discussed above are summarized in Table 1 for a better overview. Included are explicit expressions for P, and equations for $t_{0.5}$, the time needed to reach half the equilibrium concentration of the permeator, i.e. when $c_{equ}/(c_{equ} - c_i) = 2$.

To calculate P for the nonionic form of an electrolyte from data of an uptake experiment it is necessary to know the pH_i. This value is obtained from the data at equilibrium distribution of the weak acid, as outlined now for an F uptake experiment with a definite V_o: from the known total amount of F in the system (F_{total}), $c^{F^-}_i$ is calculated as:

$$c^{F^-}_{i\,(equ)} = \frac{F_{total} - V_o\,c^{F}_{o(equ)} - V_i\,c^{HF}_{equ}}{V_i} \tag{11}$$

where $c^{F}_{o(equ)}$ is measured and $c^{HF}_{equ} = c^{HF}_{i(equ)} = c^{HF}_{o(equ)}$ is calculated from $c^{F}_{o(equ)}$ and $pH_{o(equ)}$ as given in the method section. V_i has to be determined by an appropriate method. From the HENDERSON – HASSELBALCH equation pH_i results as follows:

$$pH_{i(equ)} = pK_i + \log \frac{F_{total} - V_o\,c^{F}_{o(equ)} - V_i\,c^{HF}_{equ}}{V_i\,c^{HF}_{equ}} \tag{12}$$

It may be remarked that pH_i obained from weak acid distribution represents an overall pH of the cell which is not identical with pH of a cell homogenate. It rather represents the pH of the compartments which accumulate most of

acid. The pH_i of a given compartmented cell determined with different weak acids will depend on their individual pK values, since weak acid distribution depends on pK, too. This is one of the reasons why determinations of the cytoplasmatic pH of highly compartmented plant cells with the weak acid DMO have some limits (MARIGO & al. 1982)

It has been further ignored so far that during the course of permeation of a weak acid protons also accumulate inside the plasmalemma, thus decreasing the pH difference an additionally decreasing the driving force.

This source of error may be minimized experimentally: by (I) choosing a sufficiently small solute concentration; and (II) by buffering the outer solution at least to a value which equals the buffer capacity of the cell interior.

Provided that pH_i does not change essentially during HF uptake, pH_i according to equation (12) can be used to calculate $c^{HF}_{i(t)}$ from the measured $c^{F}_{o(t)}$ values for any time during uptake by:

$$c^{HF}_{i(t)} = \frac{F_{total} - V_o\, c^{F}_{o(t)}}{V_i\,(1 + 10^{\,pH_i - pK_i})} \qquad (13)$$

It is now possible to calculate P^{HF} for any time (t) during HF uptake by means of the equation for P given in Table 1 (electrolyte, definite V_o system). Alternatively, by plotting $\ln [c^{HF}_{equ}/(c^{HF}_{equ} - c^{HF}_{i(t)})]$ versus, t, P^{HF} can be calculated from the slope according to equation (10).

This procedure requires a higher calculatory demand compared with the conventional method to determine P from the initial uptake rate. However, it has the advantage that the kinetics of uptake can be followed throughout and that deviations from the linearized function would rule out a passive diffusion mechanism of first order kinetics or indicate an interference with this process.

Results

a) Uptake experiment

A recorder trace of a typical F uptake study with turions is shown in Figure 1. At a pH 4 in the incubation medium, the F concentration (c^F_o) decreases exponentially until equilibrium is reached in about 2 hours. At pH 7 in the medium only a small uptake quite near the limit of detection is found during that time (dashed line). In both cases the medium was diluted by the adherent water and the free space water. Extrapolating the uptake curves to zero time, the amount of dilution can be determined (more exactly so in the pH 7 study), and from dilution the combined volume of both spaces. This value is listed as F-space in Table 2. Equilibrium time for F in this F-spaces is shorter than traced in the curve, because of the limiting response time of the F^- electrode, especially at smaller c^F. From experiments with higher c^F,

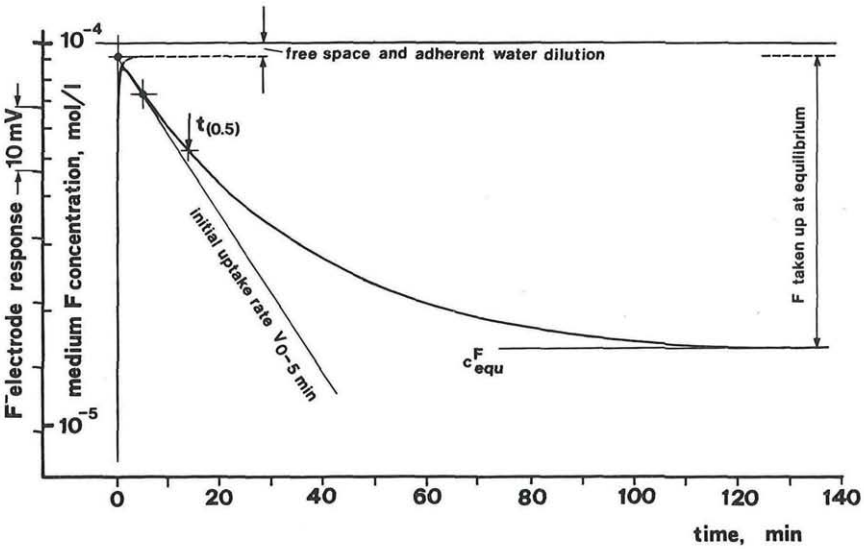

Fig. 1. Recorder trace of an F uptake experiment. Turions (2 g fresh weight) of *Spirodela polyrrhiza* were suspended in 5 ml medium buffered to pH 4. At zero time 10^{-4} mol KF/l medium was added and the decrease of F followed by the F^- selective electrode until equilibrium was reached. Dashed line: conditions as before, except medium buffered to pH 7. Arrow at $t_{(0.5)}$: half equilibrium time.

where the electrode responds faster, equilibrium time was found be below 1 minute. This indicates that the free space is also quickly accessible to F.

F uptake into turions at the same initial c_o^F depends strongly on pH: when the pH is lowered from 7 to 4, F uptake increases drastically. Uptake, therefore, cannot be a function of the F^- concentration of the medium (c_o^F). Since the concentration of the undissociated HF molecules (c_o^{HF}) increases with decreasing pH only HF can be the permeating form.

A line can be drawn with more or less accuracy along the first part of an uptake curve, whose slope is a measure of the initial uptake rate, V_0. This line can be somewhat standardized by connecting the two points at zero time and at 5 minutes ($V_{0-5\,min}$) as marked in Figure 1.

From the remaining F in the medium, the F inside the plasmalemmata can be determined according to equation (6). When V_i is known, as for turions and yeast in Table 2, also c_i^F can be determined. Turions, when separated after equilibration from the medium, dried and analysed, showed an F content within ± 5% of the F content as determined from the F taken up from the medium.

Initial uptake rates from a series of experiments with *Spirodela* turions are plotted versus c_o^{HF} at t = 2.5 min in Figure 2 (left axis). Uptake rates increase linearly with c_o^{HF}; however, in the more acid media a deviation toward

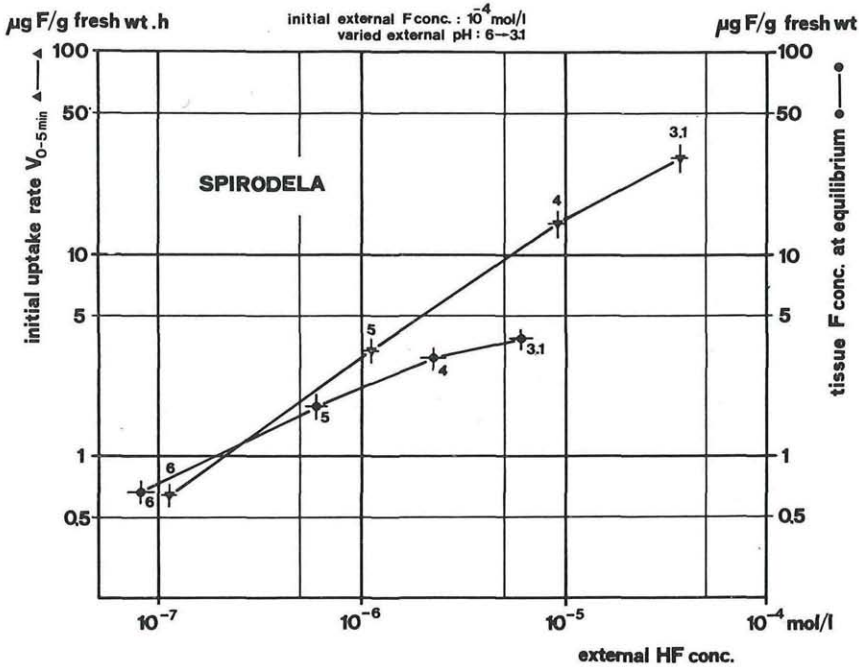

Fig. 2. Initial F uptake rates, V_{0-5min} into turions of *Spirodela polyrrhiza* (left axis, triangular symbols) and turion tissue F concentrations at equilibrium (right axis, round symbols), versus HF concentrations of the external medium, to which KF was added starting each uptake experiment. Initial external HF concentrations were adjusted by buffering the medium to different pH-values. For V_{0-5min} external HF concentration at 2.5 min is taken, for tissue F concentration the external HF concentration at equilibrium applies. Bars indicate standard error of the mean (n = 3).

lower values can be seen. The reasons for this deviation are a pH-dependent resistance in the unstirred region of the cell walls (R_{cw}) and a pH dependency of the membrane permeability coefficient (P_{pm}) itself (this will be discussed later).

Equilibrium tissue concentrations from the same series of experiments are plotted versus c_o^{HF} at equilibrium times (right axis). Tissue F accumulation is a function of both c_o^{HF} and the ΔpH between the medium and the cell interior, as is characteristic for the ion trap mechanism. In Table 3 the overall pH_i values of the cell interior at $t_{(equ)}$ are listed. It can be seen that the ΔpH values ($pH_o - pH_i$) increase with the acidity of the medium. However, since the absolute pH_i values decrease, F accumulation is relatively smaller with lower pH_i due to a smaller amount of F^- trapped inside the cells. The drop to unphysiologically low pH_i values is caused mainly by the preincubation in the acid media and to a small degree also by the uptake of HF. Preincubation is a methodical necessity to get a sufficiently stable pH_i for the evaluation of

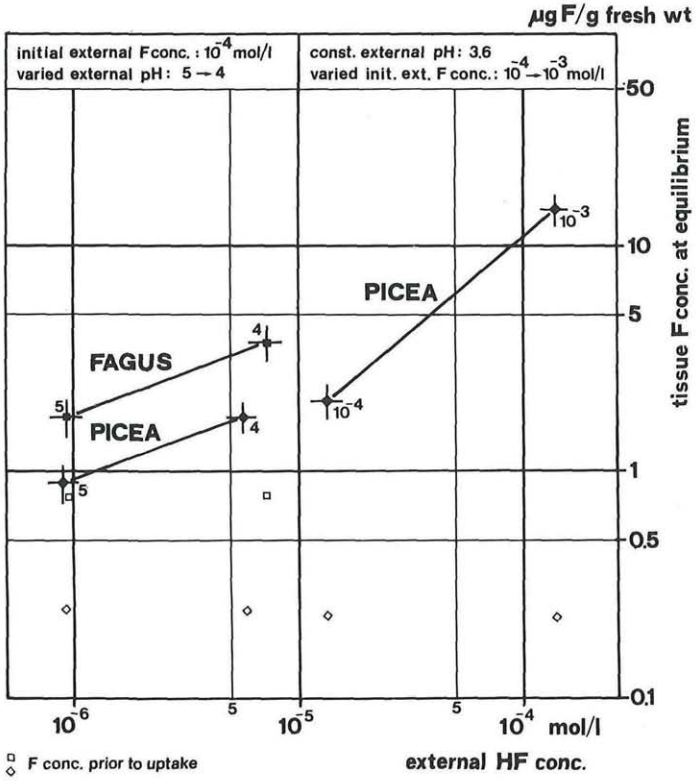

Fig. 3. Tissue F concentrations at equilibrium in leaf slices from *Fagus sylvatica* or needle slices from *Picea abies*, versus HF concentrations at equilibrium in the external medium. Left side (below 10^{-5}): initial external HF concentrations were adjusted by buffering the medium to different pH values as in Figure 2. Right side (above 10^{-5}): initial external HF concentrations were adjusted by adding different amounts of KF (at the start of each uptake experiment) to the medium, which was buffered to a constant pH. Bars indicate standard error of the mean (n = 3).

uptake kinetics as outlined in the theory section. Examples are given later in this section.

Figure 3 (left part) shows an F accumulation of leaf and needle slices similar to turions. The increases from pH 5 to pH 4 have nearly identical slopes in all the 3 species. The lower absolute accumulation in *Picea abies* is caused by the lower overall pH_i of this plant. However there are two differences compared with the turion experiments. (1) Standard errors are larger, due to a less reproducible stirring of the suspensions and probably also due to a variable amount of damaged tissue after cutting to slices. (2) In field grown leaves and needles the tissues already contained some F prior to uptake, whereas turions are virtually F-free.

On the right side of Figure 3 tissue F concentrations are given when the c_o^{HF} is varied by changing the initial c_o^F at a constant pH_o. Here the needle slices were preincubated at the same pH_o, and so the pH_i was adjusted in the same way. Therefore, F accumulates nearly 10-fold with a 10-fold increase in c_o^{HF}. It can be ruled out that the concomitant higher $c_o^{F^-}$ may contribute to a higher accumulation, as uptake even with high $c_o^{F^-}$ at pH 7 is near the limit of detection with *Picea* as well as in *Spirodela*.

Although leaf and needle slices are suitable objects for the study of HF permeation, they were not used for a quantitative determination of P, since no data are available for plasmalemma areas and protoplast volumes.

b) Measurement of the permeability coefficient

The procedure outlined in the chapter "Theory" was used to determine P values from the turion and yeast cell experiments. An example of a linearized plot of F uptake kinetics for turions is given in Figure 4. Each point represents the result of the calculation of c_o^{HF} at time (t) from the measured c_o^F and pH_o at time (t); pH_i and c_{equ}^{HF} were determined from the equilibrium data. Cell dimensions used are given in Table 2.

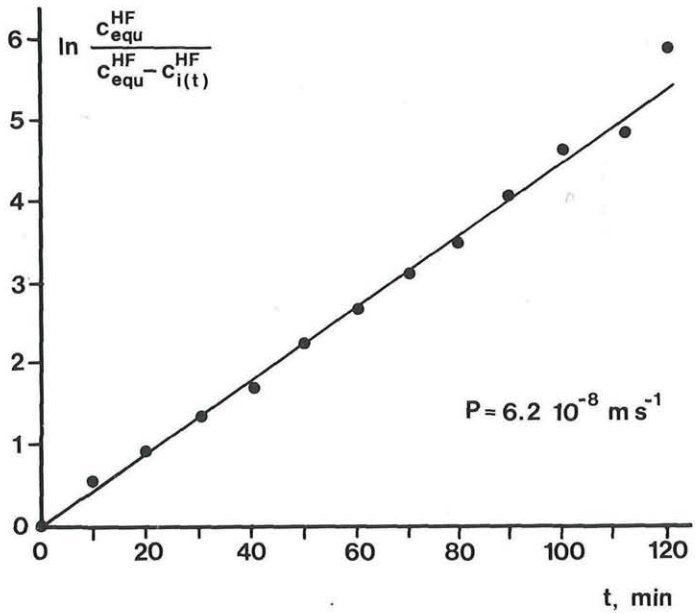

Fig. 4. Example of a linearized plot from an F uptake experiment performed with 2.0 g turions of *Spirodela polyrrhiza* suspended in 5.0 ml medium (initial F concentration 10^{-4} mol/l). Equilibrium data: pH of the medium = 4.06; overall pH of the cell interior, $pH_{i(equ)}$ = 5.15.

Table 2

Dimensions of turions and yeast cells used for F uptake experiments. Values (means ± s.e.) refer to 1 g fresh weight of both turions and yeast cell pellets as obtained after a standardized pretreatment described in the method section. Number of replicates given in parenthesis.

	turions	yeast cells
dry weight, g g^{-1}	0.345 ± 0.004 (4)	0.238 ± 0.002 (6)
specific volume, ml g^{-1}	0.969 ± 0.0018 (3)	0.920 ± 0.0005 (3)
^{3}H-dextran space, ml g^{-1}	0.124 ± 0.015 (4)	0.296 ± 0.009 (4)
^{14}C-sorbitol space, ml g^{-1}	0.212 ± 0.009 (4)	0.323 ± 0.006 (4)
F-space, ml g^{-1}	0.205 ± 0.007 (6)	0.329 ± 0.005 (6)
volume inside the plasmalemma,	0.662 ml g^{-1} *)	0.591 ml g^{-1} +
plasmalemma area,	0.242 m^2 g^{-1} *)	0.630 m^2 g^{-1} +

*) Calculated with the aid of data given by SMART & TREWAVAS (1983 b): plasmalemma area = 3263 cm^2 cm^{-3} cells, cell volume = 0.8792 m^3 m^{-3} tissue, air space volume = 0.1209 m^3 m^{-3} tissue.

+ Values are determined according to MUSFELD (1942).

It can be seen that the points fit fairly well to first-order kinetics. The last few points close to equilibrium show deviations due to errors caused by the very small changes of measured c_o^F from point to point. The first point deviates somewhat more from the line. This deviation may be explained by the delayed response o the F^- electrode for a change from the pure incubation medium to the rapidly changing $c_o^{F^-}$ at the start of an experiment. A further reason may be a small reduction of c_i^{HF}, as will be shown with yeast cells.

In Figure 5 a linearized plot of F uptake by yeast cells is given. Here a significant deviation from linearity occurs toward higher P values during a long period of uptake.

A similar deviation is obtained in turion experiments performed in an acid medium without preincubation of the turions in that medium. In such a case the experiments starts with a much higher pH_i which stabilizes only after some time during the uptake study. Thus, the higher pH_i causes a smaller c_i^{HF} compared with cells already pH_i adjusted and so a higher driving force, Δc^{HF}, results during the first time. Since for calculation pH_i is assumed to be stable, the decreasing pH_i will cause an erroneous deviation toward higher P values.

However, with yeast deviation remains even after pH_i is stabilized by preincubation. Therefore, another mechanism obviously reduces c_i^{HF} and generates a higher driving force. This mechanism was found to be a high F

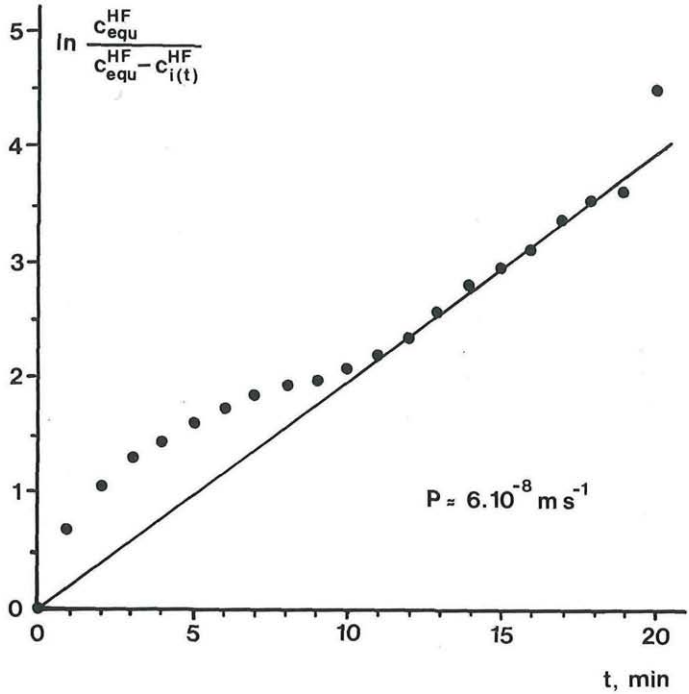

Fig. 5. Example of a linearized plot from an F uptake experiment performed with 10.0 g yeast cells suspended in 10.0 ml medium (initial F concentration 10^{-3} mol/l). Equilibrium data: pH of the medium = 4.03, overall pH of the cell interior, $pH_{i(equ)}$ = 5.31.

binding potency of the yeast cell interior. When yeast cells are frozen at −18° C, thereafter freeze dried and resuspended in a NaF solution, free F decreases in the suspension of the dead cells, as measured in terms of F^- activity. Reduction of free F depends on the c^F of the solution used. Results are shown in Figure 6. For this measurement the F^- electrode was calibrated in NaF activity standards at 25° C.

Activity is pronouncedly reduced at small c^F, has a maximum at 10^{-2} mol/l and decreases again at higher c^F. When the cell suspensions are heated to 90° C for 1 minute, brought to 25° C and measured again, F^- activity is found to increase, especially in the lower c^F region.

It can be argued that in the living yeast cell a high amount of F is bound. Thus, F penetrating into the cells will no longer exist as a free solute and will not take part in equilibration. This should lead to a higher driving force as c_i^{HF} decreases. As a consequence an erroneous deviation toward higher P values results in the same way as with a higher pH_i during the first time of an experiment. Absolute P values obtained with yeast cells will therefore contain some uncertainties, although the last part of the plot fits to a straight

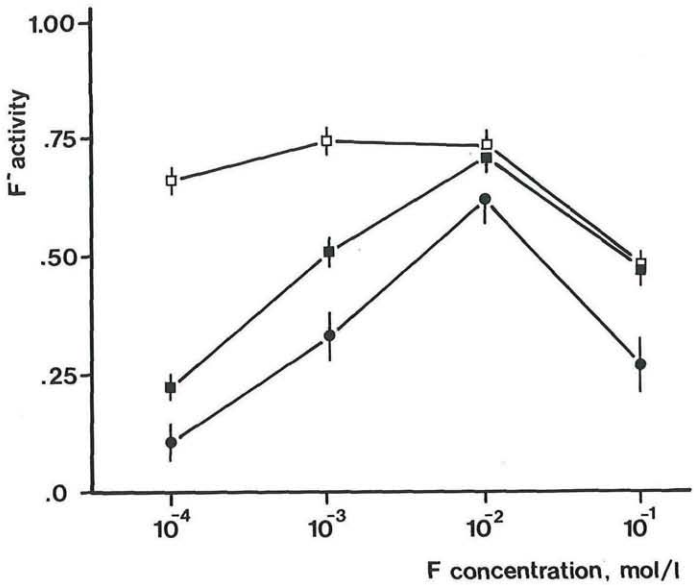

Fig. 6. Reduction of free F (measured in terms of F^- activity) in a suspension of freeze dried yeast as a function of F concentration of the NaF solution in which the suspension was prepared. Round symbols: 1 g dry yeast + 1.9 ml solution; square symbols: 1 g dry yeast + 3.8 ml solution, suspension before (closed symbols) and after heating (open symbols).

line as shown in Figure 5. A more correct value could be derived only when the kinetics of F binding could be integrated into the kinetics of F permeation.

Furthermore, it should be attempted to integrate the kinetics of the pH_i-change, caused by the pH_o as well as by the permeating acid into the kinetics

Table 3

Permeability coefficients (P) measured and P corrected for cell wall resistance (R_{cw}) for turions of *Spirodela polyrrhiza* in dependence of the pH of the medium (pH_o). Initial F concentration of the medium was 10^{-4} mol/l throughout. The overall pH of the cell interior as obtained at HF equilibrium is given under $pH_{i(equ)}$. Values are means ± s.e. (n = 3).

pH_o	$pH_{i(equ)}$	P, measured ($m\ s^{-1}$), 25° C	P, corrected for R_{cw}, ($m\ s^{-1}$)
6.0	5.96 ± 0.06	2.84 (± 0.42) 10^{-7}	2.90 10^{-7}
5.0	5.51 ± 0.04	1.46 (± 0.18) 10^{-7}	1.62 10^{-7}
4.0	5.12 ± 0.05	0.68 (± 0.09) 10^{-7}	1.15 10^{-7}
3.1	4.70 ± 0.06	0.26 (± 0.05) 10^{-7}	0.87 10^{-7}

Table 4

Permeability coefficients (P) measured and P corrected for cell wall resistance (R_{cw}) for turions of *Spirodela polyrrhiza* in dependence of the initial F concentration of the medium (c^F_o). The pH of the medium was 3.7 throughout. The overall pH of the cell interior as obtained at HF equilibrium is given under $pH_{i(equ)}$. Values are means ± s.e. (n = 3).

initial c^F_o (mol/l)	$pH_{i(equ)}$	P, measured ($m\ s^{-1}$), 25° C	P, corrected for R_{cw}, ($m\ s^{-1}$)
10^{-3}	4.82 ± 0.06	0.49 (± 0.08) 10^{-7}	1.04 10^{-7}
10^{-4}	4.88 ± 0.04	0.45 (± 0.06) 10^{-7}	0.88 10^{-7}
10^{-5}	4.91 ± 0.05	0.46 (± 0.06) 10^{-7}	0.91 10^{-7}

of weak acid permeation. This would yield more accurate results even for preincubated cells.

The P values obtained from the turion experiments are summarized in Tables 3 and 4. Permeability decreases when more acid media are used (Table 3), whereas the P value is fairly constant at a given pH_o and various initial c^F_o (Table 4). Within the last series of experiments also a c^F_o of 10^{-2} mol/l was used. However, after some time membranes were obviously damaged, when a reentry of F into the medium and loss of anthocyanin from the cells was observed.

In Table 5 results from measurements of partition coefficients with various organic solvents are given. Their suitability for predicting membrane permeabilities will be discussed.

Table 5

Partition coefficients K_{hc}, at 25° C for HF, NaF (ion pair), F^- or (NaF + F^-), between some organic solvents commonly considered as membrane models and water. Values are means ± s.e., number of replicates given in parenthesis. K_{hc} values for water after ORBACH & FINKELSTEIN (1980) and HANSCH & LEO (1979) are listed for comparison.

solvent	c^F in water (mol kg^{-1})	$F_{species}$	$K^{F_{species}}_{hc}$	K^{Water}_{hc}
hexadecane	0.1–0.5	HF	3.3 (± 0.4) 10^{-6} (4)	4.2 10^{-5}
	0.1–0.5	NaF*	2.4 (± 0.5) 10^{-5} (4)	
n-octanol	0.001–0.5	HF	2.47 (± 0.05) 10^{-1} (6)	4.2 10^{-2}
	0.05–0.5	NaF	1.52 (± 0.34) 10^{-3} (6)	
	0.05–0.5	F^-	2.81 (± 0.29) 10^{-5} (6)	
olive oil	0.1	HF	7.3 (± 0.2) 10^{-3} (3)	7.0 10^{-4}
	0.1	(NaF + F^-)	5.9 (± 0.3) 10^{-5} (3)	
ether	0.1	HF	3.6 (± 0.1) 10^{-1} (3)	1.8 10^{-2}
	0.1	(NaF + F^-)	3.2 (± 0.5) 10^{-6} (3)	

*) NaF assumed to be fully associated in hexadecane

Discussion

Results indicate that F uptake into plant cells is a process of nonionic diffusion of HF and that uptake is independent of the F^- concentration of the medium. F accumulation at equilibrium is a function of the transmembrane pH difference, since F^- becomes trapped within any compartment surrounded by a membrane due to the very low permeability for the ionic species. Thus HF behaves like other weak acids. Kinetics of uptake is of first order as shown for *Spirodela* turions.

Permeability for HF obtained from the turion experiments ("P measured" in Tables 3 and 4) is highly pH dependent, while it is independent of the HF concentration at a given pH. However, a measured P value not only represents the permeability of the plasmalemma but also contains the permeability of the cell walls (as an unstirred layer). Thus it is necessary to decide whether the contribution of the cell walls to the resulting P value is essential or not. Let us handle this question with resistances (R), the recipocals of P, as R values are simply additive: the sum of the plasmalemma resistance (R_{pm}) and the cell wall resistance (R_{cw}) is the total resistance (R_{total}) or 1/P measured.

Only an estimation can be tried, because of difficulties to measure R_{cw} of the turions independently from R_{pm}. The following dimensions of the flat and lens-shaped tissue of the turions having an average diameter of 1.7 mm were used:

turion surface area, $A^{tu} = 0.0092\ m^2\ g^{-1}$
plasmalemma surface area, $A_{pm} = 0.242\ m^2\ g^{-1}$
cross sectional cell wall area
available for diffusion into the turion, $A_{cw} = 9.7\%$ of A^{tu}
average path lenght of diffusion in the cell walls, $\Delta d = 100\ \mu m$
diffusion coefficient of HF or F^- in the cell walls, $D = 5.10^{-10}\ m^2\ s^{-1}$.

With the aid of the equation: $R_{cw} = \frac{A_{pm}\,\Delta d}{A_{cw}\,D}$, a cell wall resistance of $5.4\ 10^7\ s\ m^{-1}$ (related to A_{pm}) results. This resistance applies to both F species, because D is nearly the same for HF and F^-. As both species can diffuse through the cell walls, a "joint resistance" is to be taken into account. Details of derivation will be outlined in part II of this paper in the root uptake section. This joint resistance equals $R_{cw}/(1 + 10^{pH - pK}) = R_{cw}/x$ and is, therefore, pH dependent. With a pK of 3.1 the following R_{cw}/x values are obtained:

at pH 3.1 $R_{cw}/x = 2.70\ 10^7\ s\ m^{-1}$
at pH 3.7 $R_{cw}/x = 1.08\ 10^7\ s\ m^{-1}$
at pH 4.0 $R_{cw}/x = 6.04\ 10^6\ s\ m^{-1}$
at pH 5.0 $R_{cw}/x = 6.71\ 10^5\ s\ m^{-1}$
at pH 6.0 $R_{cw}/x = 6.79\ 10^4\ s\ m^{-1}$

These values are used to correct each P measured for its cell wall resistance according to: $R_{pm} = R_{total} - R_{cw}/x = 1/P$ corrected. The corrected P values are given besides the measured P values in Table 3 and 4. It can be seen that in the more acid media the measured P values were increasingly determined by cell wall resistances. Above a pH_o of 5 the contribution of R_{cw}/x can be neglected.

Even with the corrected P values a clear pH-dependency remains. This dependency could be somewhat altered by an error in R_{cw}/x (due to the rough estimation). Furthermore, in yeast cells, where R_{cw}/x can be neglected as it is some orders of magnitude lower, P increases with pH_o, too: at a pH_o of 4 the P value is around 6.10^{-8} m s^{-1} and at a pH_o of 5 around 10^{-7} m s^{-1}. Despite some uncertainties about the absolute correctness of the P values, their ratio should be less sensitive to errors caused by the nonlinearity of the uptake kinetics.

Membrane permeability to HF in turions and yeast cells is not constant, but decreases with decreasing pH. Whether the pH outside or inside the plasmalemma (or even both) is responsible for this behaviour cannot be decided, because in the type of experiments performed pH_i falls linearly with pH_o. Furthermore, it is not known which mechanism on the molecular level of the membrane accounts for the observed pH-dependency.

It should be remarked that a strikingly similar behaviour of the permeability for H_2O in *Spirogyra* sp. was reported by SEEMANN (1950). Here p^{H_2O} has its maximum at neutral pH values and decreases toward the acid and alkaline sides. As will be shown repeatedly in this paper this is not the sole similarity in the behaviour of HF and H_2O.

In summary it may be seen that tracing the uptake of a weak acid from a definite volume of an outer medium has some advantages. It was possible to characterize the permeation process as well as to determine quantitative P values. It was even possible from the deviation of theoretical kinetics to deduce a high F binding capacity of the yeast cell's interior.

Results given in Figure 6 indicate that F^- is bound to various cell components: F^- released after heat denaturation indicates binding to native proteins; the remaining reduction of free F^- may be caused by complexation (e.g. with Mg or Fe) and by precipitation as fluoroapatite and (especially in the high c^F region) as CaF_2; within the whole c^F range the relatively high ionic background of the yeast cell's interior is responsible for the reduction of F^- activity.

Preliminary results obtained from freeze dried leaves of various plant species showed a similar pattern of F binding. However, binding in yeast cells is exceptionally high compared with leaves.

F binding to cell components can be a mechanism of detoxication as well as the principle of toxic action by F^-. This depends on the nature of the compound or on the site to which F^- is bound. For example, binding to egg albumin as found by LUEHR & JOHNSON (1986) may be considered to detoxify,

whereas binding to enolase as studied in detail by BUNICK & KASHKET (1982) may have toxic effects due to inhibition of this enzyme. For a general concept of bound fluoride see also VENKATESWARLU (1983).

At last the absolute P values for HF should be discussed and their relationship to the hydrocarbon:water partition coefficients, K_{hc}^{HF}, in comparison with P and K values for water. P^{HF} values, corrected for cell wall resistances, are found to range from 0.87 to 2.9 10^{-7} m s^{-1} (at pH 3.1 to pH 6) for turions. P^{HF} for yeast cells is probably in the same order of magnitude. GUTKNECHT & WALTER (1981) found a P^{HF} of 3.1 10^{-6} m s^{-1} for lipid bilayer membranes (LBM) made from lecithin and a P^{HF} of 1.4 10^{-6} m s^{-1} for LBM made from a lecithin-cholesterol mixture (at 24°C, pH 4.1). The p^{F^-} for the lecithin-cholesterol LBM was found to amount 4.9 10^{-11} m s^{-1}. Thus, P^{HF} in natural membranes is about 10 times lower than in LBM.

A similar relationship exists between P^{H_2O} values for LBM and natural membranes: according to TRÄUBLE (1971), P^{H_2O} for LBM range from 0.5 to 10.10^{-5} m s^{-1} with most of the values around 2.10^{-5} and according to STADELMANN (1977) P^{H_2O} range from 1 to 15.10^{-6} m s^{-1} in a great variety of plant cells (with the exception of the *Characeae*).

Thus for both HF and H_2O, LBM are about 10 times more permeable than natural membranes, and in both types of membranes H_2O is about 10 times more permeable than HF. The second result is almost perfectly mirrored by the K values for HF and H_2O in hexadecane, K_{hd} (see Table 5): H_2O partitions into hexadecane about 10-fold more than HF. However, this ratio is reversed for the K values of HF and H_2O in the other solvents given in Table 5. Therefore, in accordance with the model solvents n-octanol, olive oil or ether, a membrane should be more permeable to HF than to H_2O. But the opposite is true and the higher P^{H_2O} is indicated only by K_{hd}. On the other hand, the K_{hc} of organic molecules in olive oil and ether are nearly as suitable as K_{hd} in predicting P values for LBM as shown by ORBACH & FINKELSTEIN (1980). But a COLLANDER plot for HF and organic molecules whould show the correct position of P^{HF} only when drawn with the K_{hd} values. Therefore, hexadecane may be favoured over the other solvents as a membrane model. From this it may further be concluded that permeation is rate limited by the apolar hydrocarbon chains within a LBM. Compared with the bulk of organic molecules HF and H_2O permeates better acording to their K_{hd}. TRÄUBLE (1971) ascribes the higher permeability for H_2O to the small size of its molecules compared with the larger organic molecules of the same K_{hd}. H_2O thus fits better into the mobile kinks of the hydrocarbon chains across the membrane. Since HF is similar in size, it could probably permeate by a "hitch-hiking" process within the kinks postulated for water. Thus P^{HF} may be lower than P^{H_2O} due to its lower K_{hd}, and higher than P of larger organic molecules of the same K_{hd} due to its small size.

A further interesting difference in K_{hc} for NaF ion pairs or ($NaF + F^-$) should be noted: whereas NaF ion pairs partition into hexadecane about 10

times more than HF the K values for HF are larger in all the other solvents. If hexadecane is a more appropriate model solvent, NaF ion pairs should permeate better than HF. Could this explain the higher root uptake of F from saline soils (DAVISON 1982), where NaF ion pairs are likely to occur in the soil solution and where the extremely low c^{HF} caused by the alkaline pH cannot be responsible for a high F uptake? Future research may solve this question.

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