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The Magnitude and Mechanism of the Passive Permeability of Cane Chloroplast Internal Membranes to Various Ions, Measured by Dielectric Dispersion

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Summary. The dielectric dispersion technique was used to investigate the permeability of chloroplast internal membranes to a variety of anions and cations. Selectivity sequences depended on the pH at which measurements were made, and results were consistent with transport via fixed charged sites in the membranes. The possibility that these charged sites could line "pores" in the membranes was also investigated. No relationship between chloroplast shrinkage and membrane permeability was apparent.

Terminology

Throughout the paper the following symbols are used:

ω = frequency of applied potential (s^{-1})
ε' = dielectric constant of chloroplast suspension
ε_0 = permittivity for free space ($F\ m^{-1}$)
σ = conductivity ($\Omega^{-1}\ m^{-1}$)
c_0 = concentration of ions in region of membranes (m^{-3})
e = ionic charge (coulombs)
ρ = volume concentration of chloroplasts in suspension
A = total area of membranes per unit volume of suspension (m^{-1})
k = Boltzmann's constant (J °K^{-1})
T = temperature (°K)

In recent papers (Gordon, 1972*a, b*) it was shown that the dielectric dispersion (ε' *vs.* $1/\sqrt{\omega}$) was linear within the region 0.5 to 50 MHz. This was attributed to electrodiffusion, that is, to the passive permeation of ions across the chloroplast internal membranes under the opposing influences of an applied electric field and a charge concentration gradient (Cole, 1968).

The slope $S_{\varepsilon'}$ of such a dispersion is given by

$$S_{\varepsilon'} \simeq \frac{3\rho^2}{(2+\rho)^2} \cdot \frac{c_0 e \sqrt{Ve}}{\varepsilon_0 A \sqrt{2kT}} \tag{1}$$

where V is the effective or apparent mobility of ions across the membranes, related to the true mobility V_t by

$$c_t \sqrt{V_t} = c_0 \sqrt{V} \tag{2}$$

where c_t is the concentration of ions within the membranes, and c_0 the concentration around the membranes.

The permeability of unit area of the internal membranes is defined as the current-to-voltage ratio, or conductance G_M per unit area (Staverman, 1952; Cole, 1968). In the above-mentioned paper it was shown that

$$G_M = \frac{c_0 e \sqrt{Ve}}{\sqrt{2kT}} \sqrt{\omega}. \tag{3}$$

If we define Φ, the "strength" of the dispersion at a particular ionic concentration by

$$\Phi_{c_0} = [(2+\rho)/\rho]^2 S_{\varepsilon'} A \tag{4}$$

then Φ_{c_0} is a good measure of the membrane permeability at a particular frequency, since from Eqs. (1), (3) and (4)

$$G_M = \frac{\varepsilon_0}{3} \Phi_{c_0} \sqrt{\omega}. \tag{5}$$

c_0 is not exactly known, since the ionic concentration around the membranes consists partly of unidentified ions carried over from the isolation process (*see* Fig. 3) and partly of ions subsequently added. Thus, the permeability to the added ions is measured in terms of $\Phi_{c_0} - \Phi_0$, where Φ_0 is the strength of the dispersion in the absence of added ions. This is a valid procedure, for provided c_0 is not too great Φ_{c_0} is found to increase approximately linearly with c_0.

From Eq. (1), linearity of Φ with c_0 implies that V is constant. As c_0 is increased, V tends to decrease, as if the membranes are beginning to saturate (Gordon, 1972*a*). In this paper, the permeabilities of monovalent and divalent ions were compared at 30 mM and 15 mM, respectively, concentrations well below saturation at all pH values investigated (Figs. 1 and 2).

By dividing a chloroplast suspension into a number of identical samples, and by adding a different ionic compound to each, permeabilities could be compared. For example, by adding sodium salts, the membrane permeability to different anions could be compared. The absolute permeability of an

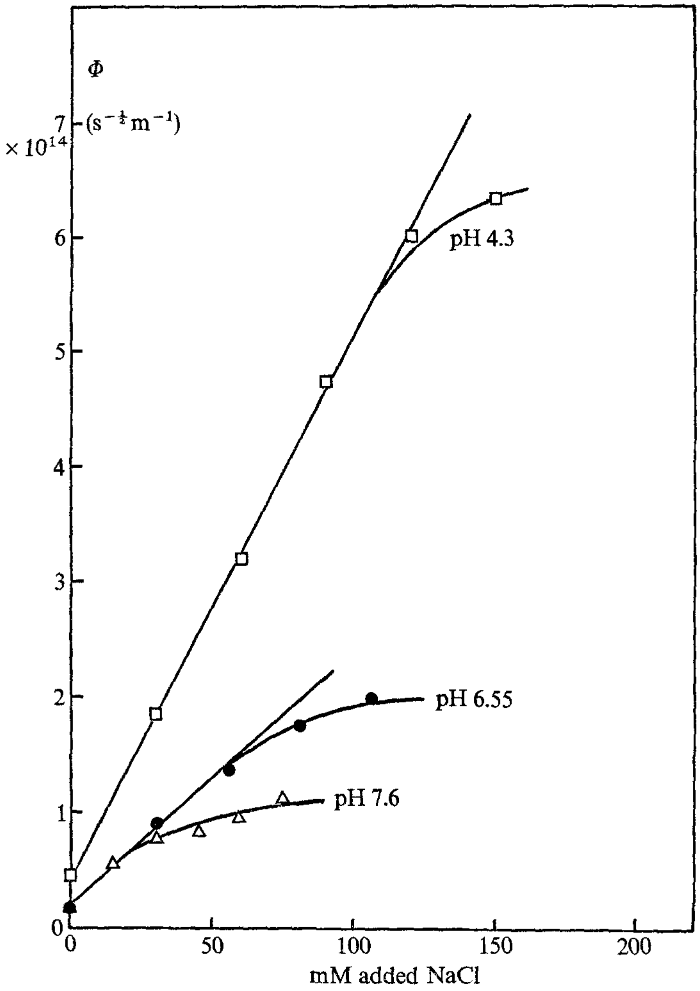

Fig. 1. Membrane saturation for NaCl at different pH values

anion could only be estimated in cases where the membrane was completely impermeable to Na^+. Similarly, the chloride salts of alkali-cations and alkaline-earth cations were compared.

Materials and Methods

Dielectric dispersions and suspension conductivities were measured in the cell described in a previous paper (Gordon, 1972*a*). The extent of electrode polarization and inductive effects was determined by replacing the suspension by its supernatant, and results were corrected according to Mandel's Eq. 6b (Mandel, 1966).

Chloroplasts were isolated from sugar cane leaves (*Saccharum officinarum*, var. unknown) as before, except that the sorbitol grinding media contained 10 mM of either β-alanine, $Na_4P_2O_7 \cdot 10\,H_2O$ or CAPS as buffer, adjusted with HCl or NaOH to give

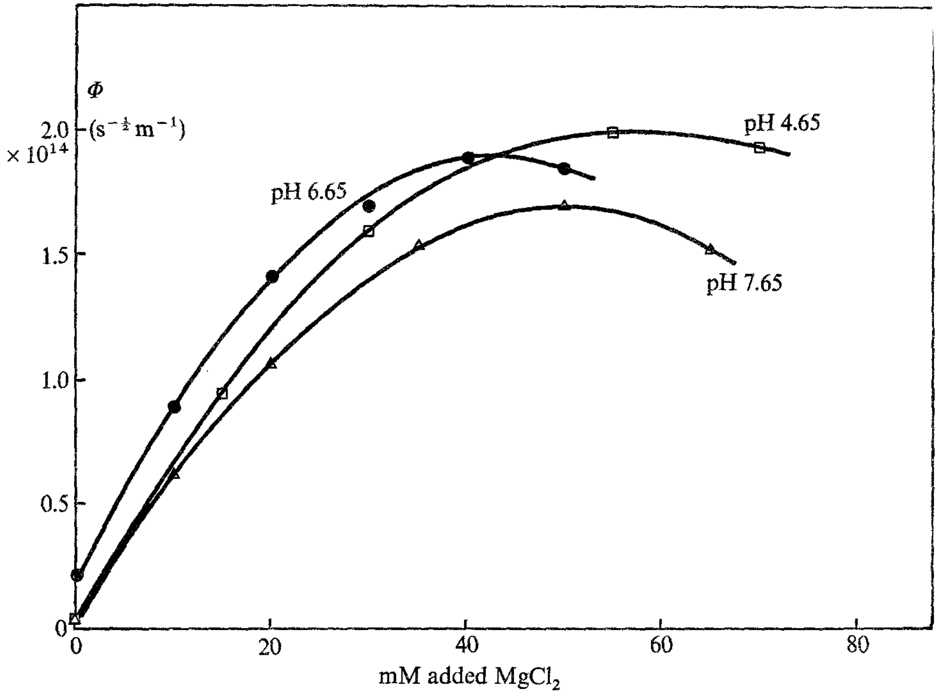

Fig. 2. Membrane saturation for $MgCl_2$ at different pH values

pH values of 3, 6.5 or 11, respectively, at 0 °C. Chloroplasts were washed and resuspended in sorbitol solution, and the appropriate ions added in solution such that the final molarity of the sorbitol in the resuspending medium was 0.32 M. Cane chloroplasts isolated in this manner were outer-membrane-free, as shown by electron-microscopy (J. Coombs, *personal communication*). The final pH of the resuspending medium (measured at 20 °C) differed from the pH of the grinding medium (*see* tables) since cane leaf tissue has itself a pH of 6 to 7.

Chloroplast volume concentrations and suspension chlorophyll were measured as before (Gordon, 1972*a*). Taking the area of membranes in a suspension to be 160 m^2/g chloroplast protein (Mitchell, 1966) and the protein to chlorophyll ratio as about 5, then a knowledge of the chlorophyll concentration enables A to be estimated.

Results

The strengths of dispersions for various ions at different pH values are given in Tables 1, 2 and 3. Data is completed with quotations of both chlorophyll and ρ/A, the latter being virtually independent of the number of chloroplasts in the suspension, and therefore a good measure of chloroplast volume.

Results for alkaline-earth cations were repeated for greater accuracy (Table 3). The repeatability of results for monovalent ions is indicated by the

Table 1. Comparison of dispersion strengths for different anions at 30 mM concentration[a]

(A) Halide anions

Grinding pH	3		6.5		11	
Final pH	4.4		6.65		8.4	
Added compound	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$
NaCl	0.967	6.66	0.617	11.53	0.656	10.29
NaBr	0.965	6.66	0.612	11.83	0.683	10.40
NaI	0.955	6.48	0.599	12.05	0.603	10.29
NaF	0.778	7.23	0.555	11.53	0.450	11.50
Φ_0	0.215	7.00	0.165	16.67	0.193	11.23
Chlorophyll (μg/0.1 ml)	108		84		113	

(B) Other anions

Grinding pH	3		6.5		11	
Final pH	5.7		6.5		8.0	
Added compound	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$
NaCl	1.439	4.89	0.543	12.70	0.494	13.06
$NaNO_3$	1.676	4.54	0.505	13.06	0.452	12.80
$NaHCO_3$	0.336	12.78	0.292	17.58	0.202	20.99
Φ_0	0.336	4.95	0.262	18.04	0.109	14.74
Chlorophyll (μg/0.1 ml)	107		123		123	

[a] Units of Φ: $s^{-\frac{1}{2}} m^{-1}$; units of ρ/A: m; e.g., for NaCl at pH 4.4, $\Phi_{co} = 0.967 \times 10^{14} s^{-\frac{1}{2}} m^{-1}$.

Table 2. Comparison of dispersion strengths for different alkali-cations at 30 mM concentration[a]

Grinding pH	3		6.5		11	
Final pH	4.4		6.5		8.1	
Added compound	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$
KCl	0.901	7.97	0.615	12.38	0.641	9.54
NaCl	0.843	7.94	0.590	12.62	0.596	9.51
CsCl	0.840	8.21	0.710	11.57	0.747	9.05
RbCl	0.865	8.18	0.693	12.03	0.716	9.21
LiCl	0.739	8.18	0.614	11.74	0.595	9.29
Φ_0	0.423	7.77	0.150	18.69	0.181	10.77
Chlorophyll (μg/0.1 ml)	177		107		152	

[a] Units of Φ: $s^{-\frac{1}{2}} m^{-1}$; units of ρ/A: m.

Table 3. Comparison of dispersion strengths for different alkaline-earth cations at 15 mM concentration, and NaCl at 30 mM concentration[a]

Grinding pH	3				6.5				11			
Final pH	4.0		4.7		6.5		6.35		7.9		7.7	
Added compound	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$
$MgCl_2$	1.43	5.25	1.41	4.49	1.43	5.60	1.53	6.35	1.29	6.13	1.04	6.55
$CaCl_2$	1.28	5.60	1.25	4.64	3.30	3.98	2.36	5.00	1.52	5.72	1.13	6.20
$SrCl_2$	1.28	5.42	1.21	4.94	3.21	3.98	1.80	5.70	1.35	6.17	0.97	6.69
$BaCl_2$	1.27	5.48	1.13	4.93	3.21	3.94	3.05	4.00	1.35	6.29	1.00	6.59
NaCl	1.403	5.48	1.313	5.10	0.798	11.16	1.115	8.90	1.296	7.68	0.761	8.66
Φ_0	0.175	5.31	0.408	4.34	0.271	16.68	0.135	16.65	0.039	9.07	0.074	10.78
Chlorophyll (μg/0.1 ml)	106		123		145		125		77		127	

[a] Units of Φ: $s^{-\frac{1}{2}}\,m^{-1}$; units of ρ/A: m.

Table 4. Repeatability of alkali-cation selectivity measurements at pH 6.5[a]

Sample	(1)		(2)		(3)		(4)		(5)	
Added compound	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$	Φ_{co} $\times 10^{14}$	ρ/A $\times 10^{-8}$
KCl	0.515	15.61	0.511	14.41	0.615	12.38	0.664	13.59	0.799	12.51
NaCl	0.477	15.74	0.472	14.70	0.590	12.62	0.591	14.09	0.711	13.10
CsCl	0.550	15.01	0.518	14.53	0.710	11.57	0.700	13.23	0.837	12.15
RbCl	0.538	14.96	0.514	14.58	0.693	12.03	0.677	13.43	0.726	12.75
LiCl	0.512	15.58	0.475	14.47	0.614	11.74	0.634	13.33	0.756	12.51
Φ_0	0.117	21.76	0.119	20.21	0.150	18.69	0.169	19.18	0.083	20.53
Chlorophyll (μg/0.1 ml)	112		111		107		189		126	

[a] Units of Φ: $s^{-\frac{1}{2}}\,m^{-1}$; units of ρ/A: m.

results of Table 4, where the alkaline-cation permeability sequence at pH 6.5 was determined on five different samples. In four out of five the same sequence $Cs^+ > Rb^+ > K^+ > Li^+ > Na^+$ was observed, and the fifth sample differed from this only in the reversal of Rb^+ and K^+. Thus, the selectivity sequences observed may be considered highly significant.

Discussion

Several theories have been put forward to explain the mechanism of ion transport through biological membranes; for example, transport via aqueous pores (Solomon, 1959) or lipid soluble carrier molecules (Wilbrandt & Rosenberg, 1961), or site mediated transfer, the sites either fixed (Teorell, 1951) or mobile (Eisenman, Sandblom & Walker, 1967).

Charged mobile sites which carry counter-ions in the form of a neutral species are not likely, since such a species would not respond to an applied electric field, and a dispersion of the electrodiffusion type would not occur, unless there were an excess of carriers. No such carriers could be detected, since in the absence of any ions in the medium, Φ was zero (by extrapolation in Fig. 3).

Fixed charged sites are a possibility, and much of the theory below is based on work with ion-exchange resins containing fixed charged sites, by means of which counter-ions are transported (*cf.* Diamond & Wright, 1969). Eisenman (1962, 1965) and Sherry (1969) predicted that only certain selectivity sequences should occur. Sequence I (the order of decreasing crystal ionic radius) is observed for weak sites, that is, sites of low electric field strength, while the reverse (sequence XI for alkali-cations) occurs for very strong sites. For charged sites between these two extremes, any of the sequences II through X may occur; the stronger the sites, the higher the sequence.

For the halide anions, sequences I through VII are predicted (Eisenman, 1965; Diamond & Wright, 1969), and similarly seven sequences are predicted for the alkaline-earth cations.

Monovalent Anions

At final pH values 4.4 and 6.65, the following permeability sequence was observed for cane chloroplast internal membranes (Table 1): $Cl^- > Br^- > I^- > F^-$ (sequence IV) and at pH values 8.4 and 7.8, respectively, the sequences $Br^- > Cl^- > I^- > F^-$ (sequence III) and $Br^- > I^- > Cl^- > F^-$ (sequence II) were observed. Thus, the transport of anions appears to be

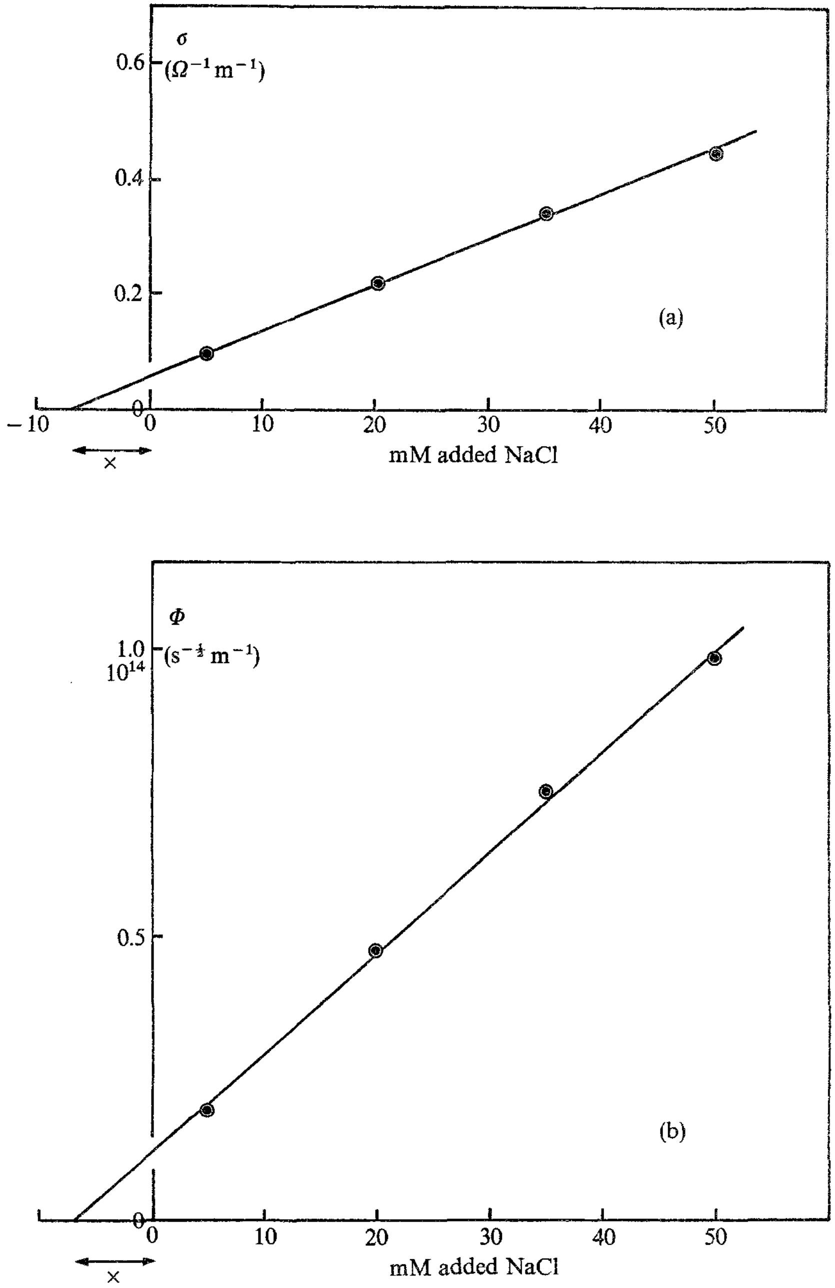

Fig. 3. (*a*) Conductivity (σ) for supernatant and (*b*) strength of dispersion of chloroplasts at different concentrations of added NaCl. Suspension chlorophyll: 111 μg/0.1 ml. x represents ions carried over from isolation process

mediated by fixed positive sites. These could well be amino groups in the membrane which would tend to be positively ionized if the membrane pH was below about pH 7, the strength of the ionization increasing as the pH was lowered.

Alkali-Cations

At a final pH of 8.1, the following order of permeabilities was observed (Table 2): $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ (sequence I). At pH 6.5, the sequence was changed by the reversal of the lithium and sodium permeabilities to give $Cs^+ > Rb^+ > K^+ > Li^+ > Na^+$ (not an Eisenman sequence) and at pH 4.4 the sequence was $K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$ (sequence V). These results are consistent with ion transport mediated by negative sites in the membrane, these sites being strongest at the lowest pH.

Although carboxyl groups tend to be negatively ionized in the membrane, they would be more strongly ionized at a *higher* pH, and are therefore unlikely to be the sites of alkali-cation transport. The lithium inversion at pH 6.5 suggests that the sites derive their charge by being polarized (Diamond & Wright, 1969). Cations could induce a negative charge in the membrane, and the greater site strength at low pH could be due to a pH gradient across the chloroplast membranes, since the chloroplast internal pH probably differs from the pH of the external medium (Dilley & Rothstein, 1967). The sites may possibly be hydrocarbons which can have a dipole induced by counter-ions in addition to their small permanent dipole, so that an effective negative charge is presented to the cations. Lithium inversion in sequence IV has also been observed for the inner surface of frog skin (Lindley & Hoshiko, 1964).

Alkaline-Earth Cations

Chloroplasts isolated at pH 3 gave the following permeability sequences (Table 3): final pH 4.0: $Mg^{++} > Ca^{++}$, $Sr^{++} > Ba^{++}$ (sequence VII); final pH 4.7: $Mg^{++} > Ca^{++} > Sr^{++} > Ba^{++}$ (sequence VII).

The sequences observed when chloroplasts were isolated in a medium of pH 6.5 were: final pH 6.5: $Ca^{++} > Ba^{++}$, $Sr^{++} > Mg^{++}$ (sequence III); final pH 6.35: $Ba^{++} > Ca^{++} > Sr^{++} > Mg^{++}$ (sequence II).

Chloroplasts isolated at pH 11 gave the following: final pH 7.9: $Ca^{++} > Ba^{++}$, $Sr^{++} > Mg^{++}$ (sequence III); final pH 7.7: $Ca^{++} > Mg^{++} > Ba^{++} > Sr^{++}$ (sequence V).

These are consistent with strong sites at low pH values (again these could be due to polarized hydrocarbons) and weaker sites at higher pH values.

However, sites appear to be stronger at pH 7.8 than at pH 6.4, so it is possible that other negative sites become important (e.g., carboxyl groups in the membrane which become more strongly ionized as the pH is increased).

Saturation

If the above theory is correct, saturation probably occurs when there are enough ions within the membranes to screen sites, thereby deterring any more ions from moving through (Diamond & Wright, 1969). The results of Fig. 1 for NaCl support this view: higher ionic concentrations are required to saturate sites as the pH is lowered, since for both monovalent cations and monovalent anions, sites become stronger.

For $MgCl_2$ the strength of sites does not vary with pH in the same manner for both anions and cations. Hence, differences in saturation are not so pronounced at different pH values (Fig. 2) although the results tend to reflect the strength of the cation sites.

One might expect to find that the membranes are most permeable when sites are strongest. This is confirmed for NaCl in Fig. 1, since $\Phi - \Phi_0$ is greatest at pH 4.3. However, the results of Fig. 2 for $MgCl_2$ do not conform to this view, since $\Phi - \Phi_0$ is greatest at pH 6.65, although sites are predicted to be weakest. Possibly at this pH the sites, although weak, are closely spaced, giving the membranes a preference for divalent cations over monovalent cations (Truesdell, 1962; Eisenman, 1965; Truesdell & Christ, 1967; Sherry, 1969). Certainly at pH 6.35 or 6.5, divalent cations were found to be significantly more permeable than sodium (Table 3).

The Possibility of Pores

Although it has been suggested that fixed charged sites account for the selectivity characteristics of the chloroplast membranes, it is possible that ions are transported through pores lined with fixed charges, for example, polarized hydrocarbons (Eisenman *et al.*, 1967; Diamond & Wright, 1969). This was investigated using the sodium salts of various monovalent anions at pH 8. At this pH, sites for both sodium and the anions are only weakly ionized, and therefore as an approximation, the membrane could be treated as if the ion selectivity were due solely to the movement of hydrated ions down aqueous pores.

The dispersion strength for $NaHCO_3$ at pH 8 was found to be very low, and therefore it was assumed (for simplicity) that sodium was excluded from the membranes.

Table 5. The hydrated radii (a) of various anions and their mobilities through sorbitol solution (v_{an}) and through chloroplast membranes (V_{an}) at pH 8[a]

Anion	v_{an} (m s^{-1}/V m^{-1}) $\times 10^{-8}$	a (Å)	V_{an} (m s^{-1}/V m^{-1}) $\times 10^{-11}$
NO_3	5.29	1.60	6.24
Cl^-	5.60	1.52	7.84
HCO^-	2.99	2.84	0.46
F^-	3.62	2.35	2.74
I^-	5.69	1.49	9.34
Br^-	5.99	1.42	9.96

[a] Sodium salts were used for which $v_{an} = 3.72 \times 10^{-8}$ and $a_{Na} = 2.28$ Å.

Let A_P/A_M = the ratio of pore-to-membrane area. Anions of large hydrated area will tend to be confined to the center of the pore, since the periphery of an ion cannot penetrate the pore "wall" (Dick, 1971). The effective pore area will be $A_P \times (R-a)^2/R^2$ where R is the pore radius, and a the hydrated ion radius. A suitable model then gives

$$V_{an} = \frac{A_P}{A_M} \frac{(R-a)^2}{R^2} v_{an}. \tag{6}$$

V_{an}, the effective mobility of anions through the membrane, was estimated from Eq. (4), and v_{an}, the mobility through 0.32 M sorbitol solution was estimated from conductivity measurements; a was calculated from Stokes' Law:

$$e = 6\pi a \eta v_{an}$$

assuming that the viscosity of 0.32 M sorbitol solution was around 1 cp (*see* Table 5).

Eq. (6) was fitted to the results using a least-squares method. This gave the curve in Fig. 4 for which $A_P/A_M = 1/211$, and the pore radius $R = 3.5$ Å (compared with 4 to 6 Å for other biological membranes; Solomon, 1968).

Since the hydrated radius of Na^+ is around 2.28 Å, this ion must be excluded from the membrane for reasons other than physical size. Even though the pore model fits the anion results, there is no conclusive evidence from the present experiments as to whether such pores actually exist.

Chloroplast Volume Changes

There was no apparent correlation between ionic permeability and chloroplast volume (Tables 1, 2 and 3) even though volume changes are thought

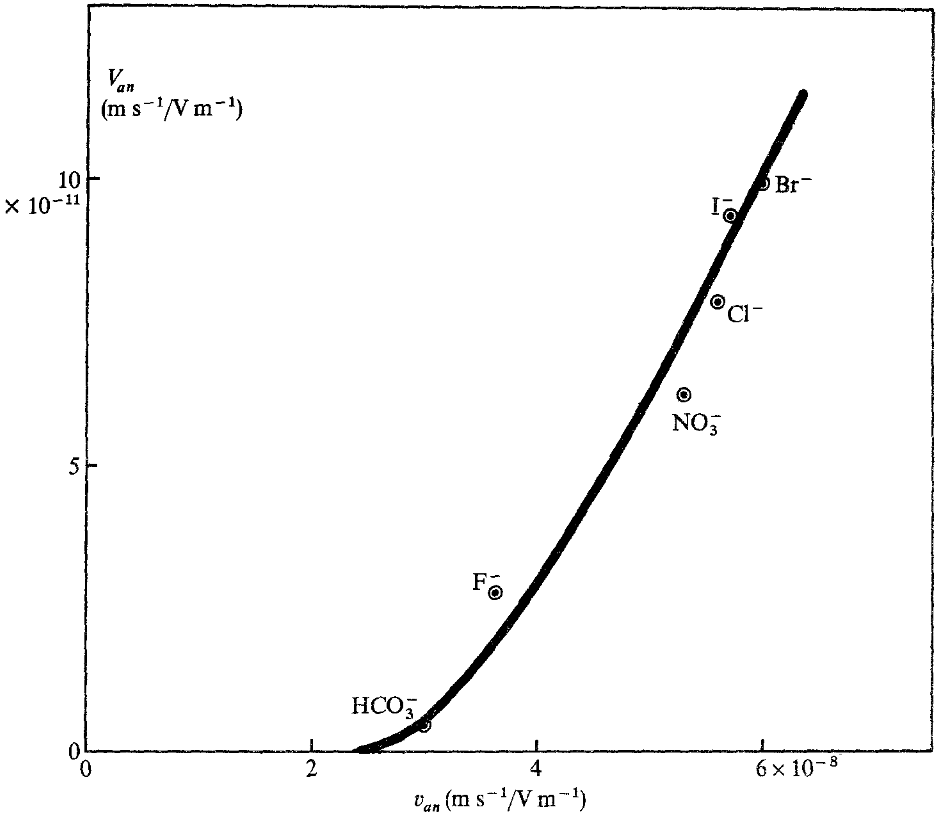

Fig. 4. Effective mobility (V_{an}) of anions through chloroplast membranes versus their mobility (v_{an}) in 0.32 M sorbitol solution. Suspension chlorophyll: 165 μg/0.1 ml

to depend on fixed charges in the membrane (Dilley & Rothstein, 1967). This implies that the same charged sites do not control both permeability and chloroplast volume.

I would like to thank Dr. C. Bucke for his advice and dicussions on this work.

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