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CHLOROPLAST MEMBRANE CHARACTERISTICS

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SUMMARY

1. Isolated *Spinacea oleracea* chloroplasts respond as reversible osmometers over limited ranges of sucrose concentration. Comparison of albumin and sucrose spaces in centrifuge tube pellets indicates that sucrose penetrates more of the chloroplast space than does albumin. Furthermore, the evidence suggests that the grana (or thylakoid) compartment is the sucrose and salt impermeable, osmotically responsive space.

2. Lowering the pH of a chloroplast suspension to the isoelectric point, *i.e.* pH 4.7, results in the same degree of shrinkage as obtained by extrapolation of the Boyle–Van 't Hoff plots. This suggests that chloroplast volume is in part determined by the degree of ionization of fixed dissociable charge groups of the membranes and possibly of non-diffusible polyelectrolytes within the chloroplast. Varying degrees of ionization of such groups would lead to changes in the amount of counter-ions within the structure, with concomitant changes in volume.

The effect of many divalent cations (Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} as chloride salts) suggests that these cations may bind to fixed groups and induce contractions similar to those obtained by lowering the pH.

3. The internal pH of chloroplasts was measured by using the distribution of a weak electrolyte (5,5'-dimethyloxazolidine-2,4-dione). It was found in general that (a) a difference in pH exists across the chloroplast thylakoid membrane with the internal pH higher than the external pH when the latter is below pH 7.1 and (b) the internal pH tends to remain near pH 7.1 as the external pH decreased to near 6.3. A pH gradient as large as 1.0 pH unit could also be induced when the external sucrose concentration was increased to 0.5–0.8 M, from 0.2 M sucrose.

INTRODUCTION

It has been recognized for some time that electron transport and phosphoryla-

Abbreviation: DMO, 5,5'-dimethyloxazolidine-2,4-dione.

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tion in spinach chloroplasts are mediated by the membrane systems which constitute the grana[1,2]. Recent studies with chloroplasts and mitochondria indicate that the energy conversation mechanisms are intimately involved in ion transport across the membrane of these organelles[3–9]. The mechanism whereby the coupling of redox energy to ion transport is not understood, but it is obvious that the physical and chemical properties of the membranes, *i.e.* osmotic and permeability behavior, Donnan effects, *etc.*, will determine the interactions mentioned above. These basic membrane characteristics have not been unequivocally determined for chloroplasts, though a considerably literature on these topics has accumulated[3–6, 10–17].

This report will present some results of our studies on the osmotic response of spinach chloroplasts, Donnan distributions of Rb^+, Na^+, and Cl^- as a function of pH and ionic strength, and the estimation of the internal pH of plastids determined by the distribution of a weak electrolyte. The data will be discussed in the context of recent work which has revealed some of the relationships between electron transport and photophosphorylation on one hand, and ion transport and volume changes on the other.

METHODS

Spinach chloroplast preparations and chlorophyll assay were carried out as described in ref. 18. Light-scattering measurements were made as outlined in ref. 18.

Packed-cell volume was measured in microcapillary tubes after centrifugation for 20 min in an International Model MB centrifuge. Control experiments indicated that plastids reached 90–95% of the final packed-cell volume after this time of centrifugation. Free space in the pellets was determined by measuring the content of [^{131}I]albumin (Squibb Co.) in the pellet. The albumin concentration was sufficiently high to saturate reversible binding sites, for the addition of varying amounts of unlabeled albumin did not change the [^{131}I]albumin distribution. The ^{131}I γ emission was measured by a NaI crystal detector and recorded with a Nuclear-Chicago scaler. A diamond-dust abrasive disc attached to a small electric motor was used to cut the microcapillary tubes at the interface, allowing the counting of pellet and supernatant separately. The same technique was used to study the distribution of ^{86}Rb, ^{36}Cl, [^{14}C]sucrose and ^{14}C-labeled 5,5'-dimethyloxazolidine-2,4-dione (DMO) using a Nuclear-Chicago end-window β counter.

Titration of chloroplast suspensions was carried out on a Beckman Model 9600 pH meter. The internal pH of chloroplast grana was calculated from measurements of the distribution of a weak acid, [2-^{14}C]DMO*. This compound has been used extensively for the measurement of intracellular pH in various animal tissues[19]. The method involves determination of total DMO within the cellular water space and the accurate measurement of the external DMO and pH. Knowing the pK of the acid (pK = 6.13 at 37° at I = 0.16), and assuming (a) that the undissociated form is in equilibrium across the membrane and (b) that the pK is the same in the cell as in the external medium, one may calculate the pH in the cell from the Henderson–Hasselbach equation, *i.e.*, $pH_i = pK + \log [A^-]_i/[HA]_i$; where $[HA]_i$ is assumed equal to $[HA]_0$ and $[A^-]_i$ is obtained by difference.

*[2-^{14}C]DMO was obtained from the New England Nuclear Corporation.

RESULTS

Volume–osmotic pressure relationship

A cell surrounded by a semi-permeable membrane will in general respond to osmotic pressure in accordance with the Boyle–Van 't Hoff law, *i.e.* $V - b = 1/\Pi RTn_2$; where V = total cell volume, b = non-solvent volume of the cell (volume of cell solutes *plus* solids such as cell membrane *plus* any other osmotically unresponsive compartments), Π = osmotic pressure, n_2 = number of solute molecules within the volume being measured, R = gas constant, and T = absolute temperature[20]. The equation indicates that a plot of V *vs.* $1/\Pi$ will yield a straight line with the y-intercept at b and having a slope of RTn_2 (a constant unless solutes enter or leave the cell). As a first approximation, the osmolarity c, may be used in place of Π. Fig. 1a shows

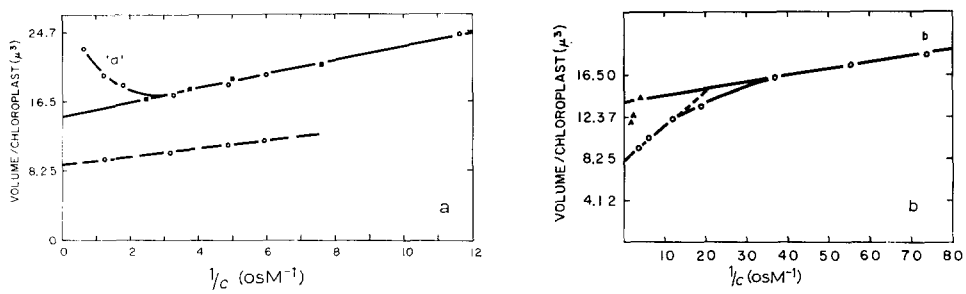


Fig. 1a. Reversible osmotic volume changes of chloroplasts. Reaction mixtures contained the following: 1 mM Tris acetate (pH 7.0), 0.504 mg chlorophyll equivalent chloroplasts, and various concentrations of sucrose. The ordinate represents the per cent of the total volume in the microcapillary tube as pellet, with no correction for free space. The abscissa is the reciprocal of the total osmolarity of the solution, with the contribution from sucrose contained in the stock chloroplast suspension calculated from vapor pressure osmometer measurements of supernatant from a sample of stock plastids which were centrifuged (total osmolarity of stock solution was found to be 0.11 osM) Circles depict the shrinkage phase and the squares represent the swelling of the sample marked 'a'. A correction factor was applied to the diluted samples to account for the effect of dilution on the packed-cell volume. The dashed line is that obtained when a free space correction factor is applied; see text for details.

Fig. 1b. Osmotic volume changes over a wide range of osmotic pressure. Reaction mixtures were similar to those of Fig. 1a. The sample denoted by 'b' was placed in more concentrated sucrose solutions to check on the reversibility of swelling, these data being given by the triangles. A correction factor was applied to the diluted samples as in Fig. 1a.

a representative chloroplast experiment in which it is seen that a linear plot obtains over a range of sucrose from about 0.4 to 0.08 M. The deviation from linearity in the left-most three points is primarily due to a larger free space which occurs in all "chlorocrits" at high sucrose (or NaCl) concentrations, and partly due to a variable anomalous swelling which may take place under such circumstances. After correction for free space (using [^{131}I]albumin) these points fall very close to a linear plot as seen by the lower set of points in Fig. 1a. In a pellet from 0.05 M sucrose to 0.3 M sucrose the free space was in the range of 38–48%, while that in sucrose concentrations from 0.5 M and up was as high as 50–55%.

Aliquots of the sample denoted 'a' in Fig. 1a were diluted to varying degrees with water to check on the reversibility of the chloroplast shrinkage. The plastids

did swell along the same line that the shrinkage curve followed, indicating that plastid shrinkage over this range of tonicity is reversible.

Fig. 1b shows the osmotic response of plastids to lower tonicities. In the neighbourhood of 0.05 M sucrose the curve gradually deviates from the linear response seen at higher concentrations and establishes a second linear region with a lesser slope. In this region the osmotically responsive volume ($V - b$) did not double in response to a halving of the osmotic pressure as occurs in the linear portion in the higher range of sucrose concentration, but experienced only a 1.75-fold swelling. MERCER *et al.*[15] have shown a similar plot for Nitella chloroplasts with the further refinement of having microscopic evidence showing that the change in slope of the plot coincides with the onset of intralamellar swelling. The swelling in 0.016 M sucrose in the present experiment was largely irreversible (as seen by the triangles in Fig. 1b), and the value of b, the osmotically unresponsive volume was increased.

An estimate of the individual chloroplast volume was made by assuming a value for the average chlorophyll content of spinach chloroplasts. The ordinates of Figs. 1a and 1b give the values calculated from TOLBERG AND MACEY's paper[11], *i.e.* 1 mg chlorophyll $= 1.5 \cdot 10^9$ chloroplasts or $4.4 \cdot 10^8$ chlorophyll molecules per chloroplast. The b values from Figs. 1a and 1b are 9.0 and 8.2 μ^3, respectively. If the value for chlorophyll content per chloroplast given by THOMAS, MINNAERT AND ELBERS[21] is used *i.e.* $1.3 \cdot 10^9$ chlorophylls per chloroplast, the b value of Fig. 1b is 25 μ^3. The values of TOLBERG AND MACEY[11] appear to give a more realistic number for the b value, inasmuch as a literature value of 30 μ^3 for an average chloroplast volume (freshly isolated) in isotonic medium is given by RABINOWITCH[22], and it follows that the non-solvent volume (b) would be considerably less than 30 μ^3.

ITOH, IZAWA AND SHIBATA[23] have made careful measurements of chloroplast volumes using the Coulter counter, and they found that the size of freshly isolated spinach chloroplasts in 0.35 M NaCl is 23 μ^3. This is the peak of the volume distribution curve. They report that an average granum is about 0.05 μ^3 in volume, and assuming 140 grana per chloroplast, they arrive at a total grana volume of 7 μ^3 per chloroplast. This volume is 30% of the total chloroplast volume at 0.35 M NaCl.

The volume–osmotic pressure relationship in various salts was compared to that in sucrose. It was found that the plastids obeyed the Boyle–Van 't Hoff law in NaCl,

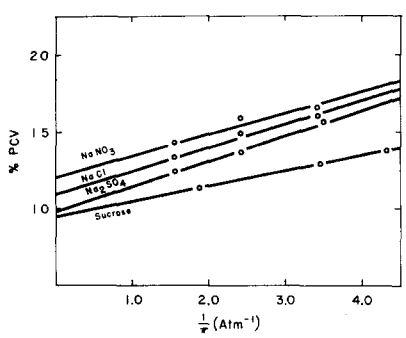


Fig. 2. V *vs.* $1/c$ relationship for chloroplasts in sucrose, NaCl, Na_2SO_4, and $NaNO_3$. The reaction mixtures and conditions were similar to those of Fig. 1, except various salts were substituted for sucrose. PCV, packed-cell volume.

KCl, $NaNO_3$, and Na_2SO_4; but there was a significant degree of swelling in all the salts compared to the volume in sucrose (see Fig. 2). The slope of the V *vs.* $1/c$ plots in salts is greater than in sucrose, indicating that a larger osmotic compartment is responding to the salts. The b values for the sucrose and salt plots were not significantly higher after 75 min than they were at 10 min (the time the centrifugations were begun). This behavior suggests that the plastids undergo a gel-type (Donnan) swelling in response to salts. The order of salt swelling effect is: $NaNO_3 > NaCl > KCl > Na_2SO_4$.

Sucrose and albumin distributions

The distribution of [^{14}C]sucrose and [^{131}I]albumin in centrifuged chloroplast pellets indicates that sucrose penetrates a much larger portion of the plastid structure than does albumin (see Table I). Albumin occupies about 45% of the pellet volume

TABLE I

COMPARISON OF SUCROSE AND ALBUMIN SPACE IN CHLOROPLAST PELLETS

[^{14}C]Sucrose and [^{131}I]albumin space were measured as described under METHODS with the microcapillary tube technique.

pH_o	*Sucrose (M)*	*Per cent sucrose space* per cent packed-cell volume (× 100)	*Per cent albumin space* per cent packed-cell volume (× 100)	*Per cent chloroplast as sucrose space*
7.0	0.05	84	45	71
	0.2	84	45	71
	0.5	99	45	98
8.5	0.05	80	44	64
	0.2	92	46	85
	0.5	94	44	89

over a wide range of sucrose concentration and at pH values of 7.0 to 8.5. Sucrose space, on the other hand, is a function of both tonicity and pH, decreasing with a lowering of the osmotic pressure or with an increase in pH, an inverse relationship to chloroplast swelling in each case. The decrease in sucrose space as chloroplasts swell is expected if an osmotic compartment impermeable to sucrose and salts does indeed exist as suggested by Figs. 1a and 1b, and Fig. 2. The constancy of the albumin space on the other hand suggests that the albumin is not distributed within some compartment which is permeable to sucrose and salts. It is possible that the albumin, but not sucrose and salts, is withheld by a portion of the grana membrane space or by a gel phase. In either case, a comparison of the sucrose or salt penetrated space to the albumin space of a pellet serves as an experimental tool to delineate two compartments in isolated chloroplasts, only one of which appears to be impermeable to sucrose and salts.

Interpretation of slope and intercept of Boyle–Van 't Hoff plot

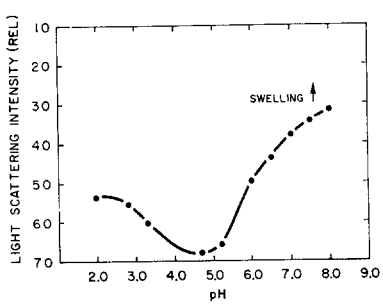
Knowing the non-solvent volume, the free space, and the slope of the V *vs.* $1/\Pi$

plot, one may calculate how much of the plastid structure constitutes the osmotic compartment at any particular solute concentration. Such calculations show that the plastids used to obtain the data of Fig. 1a and 1b had osmotic compartments comprising 25 and 29% of the total plastid volume (defined as albumin excluded volume) at 0.25 M sucrose. This is in good agreement with the estimation of the osmotic compartment from the sucrose and albumin data, which indicates that sucrose penetrates about 75% of the chloroplast space under these conditions.

Since the slope of a V *vs.* $1/\Pi$ plot is given by RTn_2 where n_2 is the total number of solute particles in the total osmotic volume being measured, it is possible to use a change in slope between two linear portions of Fig. 1b as an indicator of the relative osmotic volumes involved. The ratio of the two linear slopes of Fig. 1b is 5.7, therefore, the osmotic volume involved in the swelling in the more dilute sucrose is only 18 ($1/5.7 \times 100$) % of the total osmotically responsive volume. This analysis indicates that a large portion (over 80%) of the osmotically responsive space either bursts or becomes leaky to sucrose at tonicities below 0.05 M.

pH dependence of volume–osmotic pressure behavior

Nitella chloroplast volume has been shown to be pH dependent by MERCER, *et al.*[15], using microscopic observation. We have found a similar behavior for spinach chloroplasts from light-scattering studies (Fig. 3), with a volume minimum near pH 4.7, a value close to the isoelectric point for chloroplasts from many species[15,24]. On returning the chloroplasts from pH 4.7 to higher values of pH, they regain over



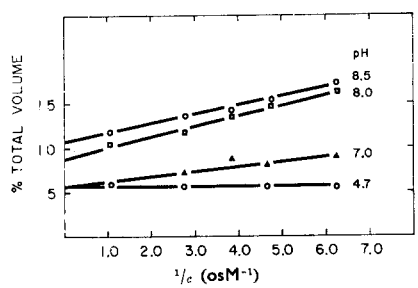


Fig. 3. Effect of pH on volume of chloroplasts. Reaction mixtures contained 0.1 M sucrose, and 0.966 mg chlorophyll as chloroplasts, in 20 ml total volume. The pH was adjusted with 0.01 M HCl and a 3-ml aliquot of plastid suspension was taken at intervals for light-scattering measurements.

Fig. 4. Effect of pH on chloroplast osmotic response. Reaction mixtures contained 0.242 mg chlorophyll as chloroplasts, 0.05 M Tris–HCl buffer (except the pH 4.7 series which were in acetate buffer, 0.05 M), and sucrose at various concentrations in a total volume of 0.2 ml. Packed-cell volume was used as a measure of chloroplast volume.

75% of their former volume as measured by light scattering. DEAMER AND PACKER[25] also found that the acid-induced light-scattering increment was reversible.

The effect of pH on chloroplast volume as a function of external osmotic pressure using the 'chlorocrit' technique is illustrated in Fig. 4. At their isoelectric point (pH 4.7), chloroplasts swell very little, if at all, as the tonicity of the suspension is lowered. This behavior suggests that at pH 4.7 the osmotic compartment may

have lost most or all of the solutes which are present at higher pH values, resulting in an osmotic loss of water and thereby accounting for the shrinkage. This interpretation is corroborated by studies of ion distribution described below (Table II and Fig. 7) in which it is found that Rb^+ is concentrated in the plastid at pH 7.0 by a factor of nearly 2-fold over the external concentration, while at pH 4.7 there is

TABLE II

$^{86}Rb^+$ AND $^{36}Cl^-$ CONCENTRATION RATIO BETWEEN CHLOROPLASTS AND SUSPENDING MEDIUM
Conditions were the same as for Fig. 6.

pH	Rb_i^+/Rb_o^+	Cl_i^-/Cl_o^-
4.7	1.20	1.00
7.0	1.62	1.00
8.5	1.60	0.95

considerably less Rb^+ in the plastid. The increase in the slope of the V *vs.* $1/c$ plot as the pH is increased from 4.7 to 7.0 (Fig. 4) indicates that the osmotic compartment has regained solute at the higher pH, leading to an increase in the term RTn_2 contained in the Boyle-Van 't Hoff expression. The constancy of the intercept indicates that the size of the osmotically unresponsive volume has not changed. Above pH 7.0, however, both the slope and intercept of the plots are increased. In addition to a further increase in the solute content of the osmotic compartment, the non-osmotic compartment is also enlarged presumably because of a gel-type swelling. Table I shows that pH 8.5 conditions lead to an increase in the sucrose impermeable space, consistent with the greater slope of the volume–osmotic pressure curve at pH 8.0 and 8.5 compared to pH 7.0.

Effect of divalent cations on chloroplast volume

The use of divalent cation interaction with proteins has been a useful experimental tool for the study of ionizing groups of proteins. BRESLOW AND GURD[26] found

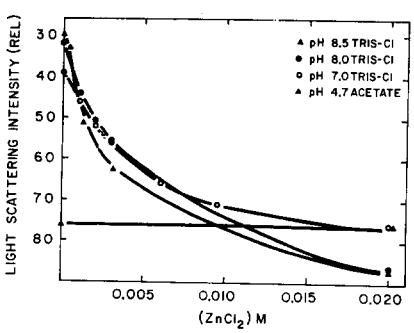


Fig. 5. $ZnCl_2$ effect on chloroplast volume. Relative plastid volume was measured by 90° light scattering. The reaction mixtures contained 0.966 mg chlorophyll, and 0.1 M sucrose, 0.05 M buffer (Tris–HCl or acetate) in 20 ml total volume. $ZnCl_2$ was added to 3-ml aliquots to make a series of $ZnCl_2$ concentrations (one series was run with $MnCl_2$). The triangle between 70 and 80 on the ordinate represents data using pH 4.7 acetate buffer.

TABLE III

EFFECT OF VARIOUS DIVALENT CATIONS ON CHLOROPLAST VOLUME

Reaction mixtures contained 0.1 M sucrose, 0.05 M Tris–HCl (pH 8.5), 0.437 mg chlorophyll equivalent chloroplasts, and 0.02 M salt as indicated in a total volume of 0.2 ml. Relative chloroplast volume was measured by packed-cell volume and recorded as per cent of total volume as pellet.

Salt added	*Packed-cell volume (% ± S.D.)*
None	27.7 ± 0.1
$MgCl_2$, 20 mM	20.9 ± 0.1
$MnCl_2$, 20 mM	12.7 ± 0.1
$Co(NO_3)_2$, 20 mM	12.2 ± 0.1
$CuCl_2$, 20 mM	11.5 ± 0.1
$NiCl_2$, 20 mM	11.9 ± 0.1

that Zn^{2+} and Cu^{2+} interact with the imidazole groups of metmyoglobin, resulting in conformational changes similar to those induced by H^+. Fig. 5 shows the effect of $ZnCl_2$ on plastid volume at several pH values. At pH 7.0 the Zn^{2+} causes the plastid volume to shrink to the 'isoelectric' (pH 4.7) volume. At pH values of 8.0 and 8.5 Zn^{2+} causes a greater shrinkage than pH 4.7 exposure, as measured by light scattering.

Several divalent cations were tested for their effects on chloroplast volume when the suspension was held at pH 8.5 using the packed-cell volume technique. Table III shows that $MnCl_2$, $Co(NO_3)_2$, $CuCl_2$, and $NiCl_2$ (all at 0.02 M) were equally effective in reducing the volume by about 50%, while $MgCl_2$ at 0.02 M reduced the volume only 25%. Separate experiments showed that these salts at 0.02 M did not alter the pH of 0.05 M Tris–HCl with or without chloroplasts present. Fig. 6 shows the relative effect of $CaCl_2$ and $MnCl_2$ on the volume–osmotic pressure relationship at pH 7.0, 8.0 and 8.5. $CaCl_2$ at 0.02 M decreases the volume of chloroplasts at pH 8.5 slightly below the usual volume (see Fig. 3 for comparison), but the effect is not at all as pronounced as that of $MnCl_2$, which drastically inverts the order of the pH effect on volume. Monovalent ions such as NaCl, KCl, and Na NO_3 do not affect chloroplasts as do Zn^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}.

The effect of $ZnCl_2$ in shrinking chloroplasts was just as pronounced with

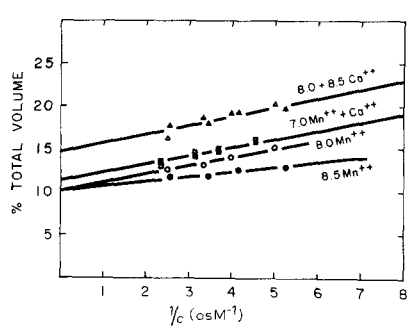


Fig. 6. $MnCl_2$ and $CaCl_2$ effect on plastid volume. Volume measurements were made by packed-cell volume. The reaction mixtures contained 0.446 mg chlorophyll, 0.05 M Tris–HCl buffer at various pH values, $MnCl_2$ or $CaCl_2$ at 0.02 M, and water up to total volume of 0.2 ml. Sucrose was used to vary the osmotic pressure.

subchloroplast particles. For this experiment, particles were prepared from chloroplasts given a 30-sec sonication (at 5-A current) in a Bronson instrument. Particles which sedimented between 20 000 × *g* and 50 000 × *g* and the supernatant from the 50 000 × *g* centrifugation were used. Essentially the same results of light scattering intensity *vs.* $ZnCl_2$ concentration were obtained as depicted in Fig. 5 for unsonicated plastids. The supernatant from the 50 000 × *g* centrifugation was equally responsive to $ZnCl_2$, however, this fraction contains stroma protein as well as small lamellar particles so one cannot be sure which component was contributing to the light-scattering signal. These data are consistent with the notion that ionizable groups of intact plastids and the grana lamellae system can interact with H^+ and certain metal ions and that such interaction can lead to changes in volume. The fact that the maximum effectiveness of Zn^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+} is not observed until pH 8.0 to 8.5, may indicate that groups which ionize in that pH range could be involved in plastid volume control.

Rb^+ and Cl^- distribution ratios

MERCER *et al.*[15] concluded that Nitella chloroplasts have a Donnan system of fixed negative changes at pH values above 4.7. If a Donnan system of this type occurs in spinach chloroplasts, it would be indicated by a greater concentration of permeable cation (Rb^+ in this case) in the chloroplast compared to the external solution. In a perfect Donnan system the mobile anion ratio should be the inverse of the mobile cation ratio, *i.e.* $Rb_i^+/Rb_0^+ = Cl_0^-/Cl_i^-$. Table II shows the effect of pH on the distribution ratios of Rb^+ and Cl^-. The cation concentration is considerably

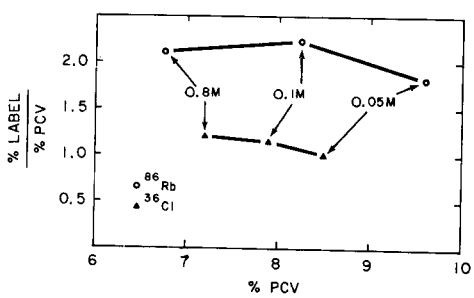


Fig. 7. $^{86}Rb^+$ and $^{36}Cl^-$ distribution between chloroplasts and suspending medium. Reaction mixtures contained 1.07 mg chlorophyll equivalent chloroplasts, 0.05 M Tris–HCl buffer at pH 7.0, sucrose at various concentrations, and $^{86}RbCl$ or $Rb^{36}Cl$ in a total volume of 0.6 ml. Centrifugation was carried out in microcapillary tubes as described in METHODS. The ordinate gives the ratio of per cent of total label in the chloroplast volume to the per cent volume as chloroplast space (both values were corrected for the free space). The abscissa gives the chloroplast volume in the pellet corrected for free space. PCV, packed-cell volume.

increased in the pellet as the pH is increased from 4.7 to 7.0, while the anion concentration is not measurably affected. Na^+ showed a similar distribution pattern as Rb^+ at pH 7.0. It is important to note that, although the internal Cl^- concentration does not increase as the pH is changed from 4.7 to 7.0, the absolute amount of Cl^- in the chloroplast must increase significantly, since the volume increases by a factor of about 1.25.

Fig. 7 shows the effect of the osmotic volume relationship on the $^{86}Rb^+$ and

$^{36}Cl^-$ distribution ratios at pH 7.0. The data are presented as a ratio of the per cent label space to per cent packed-cell space, plotted against the packed cell space (both corrected for free space). Rb^+ is concentrated in the plastid by about a factor of two, but Cl^- is much less concentrated. As the plastids shrink by increasing the sucrose from 0.1 to 0.8 M the Rb_i^+/Rb_0^+ ratio decreases while the Cl_i^-/Cl_0^- ratio increases, but both species are diluted as the plastids swell by lowering the sucrose concentration from 0.1 to 0.05 M. It is evident from these data and those of Table II that the anions and cations are not responding to the same fixed charges in the chloroplast. At least one compartment must be impermeable to one species of ion.

Internal pH of chloroplasts

The distribution of the weak acid, DMO, between the chloroplasts and the medium can be used to estimate the internal pH, provided that only the undissociated form of DMO can penetrate the membrane and provided that binding is not extensive[19]. In the absence of binding the distribution ratio (inside to outside) should be independent of DMO concentration. Such is the case for concentrations above 0.7 mM. At lower concentrations, however, the ratio decreases along an asymptotic curve, indicating that a limited amount of binding that is saturated at 0.7 mM DMO, contributes to the distribution. From the data the maximum binding can be estimated to be less than 70 μmoles/l of chloroplast water. At the concentration of DMO used for determining pH, 1.7 mM, the error due to binding is less than 5%.

If the chloroplast membranes were permeable to both the undissociated

TABLE IV

DMO DISTRIBUTION AT VARIOUS pH VALUES

Reaction mixtures contained the following: at pH 4.7 and 5.5 acetate buffer at 0.1 M, at pH 7.0, 0.1 M Tris acetate, 0.866 mg chlorophyll equivalent chloroplasts, 1.7 mM [2-^{14}C]DMO in a total volume of 0.4 ml. See METHODS for details of technique.

pH	*Per cent DMO space*
	per cent packed-cell volume
4.7	1.32
5.5	1.21
7.0	0.94

DMO and DMO ion, then the distribution coefficient of DMO in chloroplast water should be 1.0 at all values of external pH (or 1.05 if the binding is taken into account). Actually the distribution ratio responds to external pH (Table IV). At pH 4.7 the ratio is 1.32 and at pH 7.0 it is 0.94. The direction of the changes is consistent with the hypothesis that at least part of the chloroplast volume is bounded by a membrane permeable to the undissociated DMO but not to the ionic form. The calculated values for internal pH indicate that the pH within the chloroplast is influenced by external pH, but that at low values of external pH, the pH inside the chloroplast is considerably higher (Table IV). Additional experiments (Table V) suggest that the internal pH increases as the tonicity is increased.

TABLE V

INTERNAL pH OF CHLOROPLASTS MEASURED BY DMO DISTRIBUTION

See METHODS for discussion of technique. Reaction mixtures and method of handling were similar to that described in Fig. 7.

Expt.	*Sucrose (M)*	pH_o	pH_i
A	0.1	7.38	7.54
			7.55
	0.3	7.38	7.47
			7.48
	0.5	7.38	7.98
			8.33
B	0.05	6.30	7.02
	0.2	6.36	7.08
	0.8	6.30	7.29
C	0.05	6.62	7.13
	0.2	6.62	7.18
D	0.05	7.2	7.17
	0.1	7.2	7.22

The values for internal pH in Tables IV and V are calculated on the basis that the chloroplast is a single compartment bounded by a membrane permeable only to undissociated DMO. This is obviously an oversimplification. The compartment or compartments impermeable to dissociated DMO are probably only a fraction of the total chloroplast and may be identical with the osmotically responsive compartment. In this case the differences between external and internal pH could be considerably greater than shown in Table V. The calculated values of internal pH cannot therefore be used in a quantitative sense. They do strongly indicate, however, that the pH in some compartments in the chloroplast is not the same as that in the medium, and that this difference is influenced by the external pH and by the tonicity.

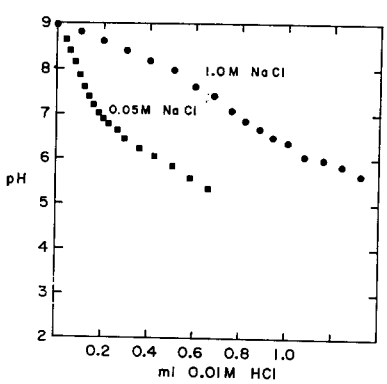


Fig. 8. Effect of NaCl concentration on pH titration of chloroplasts. Reaction mixtures contained 0.181 mg chlorophyll as chloroplasts, 0.05 M or 1.0 M NaCl, and water up to 15 ml total volume. The 0.05 M NaCl treatment required 0.40 ml 0.01 M NaOH to adjust the pH to 9.00, and the 1.0 M NaCl treatment required 0.98 ml 0.01 M NaOH to reach pH 8.95 (both reactions had an initial pH of 6.62).

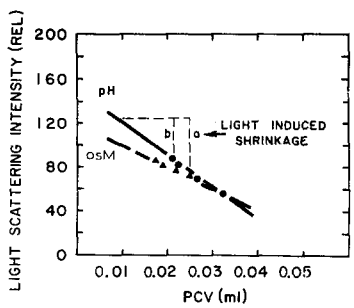
Such behavior would be expected of a compartment containing dissociable fixed charges, bounded by an ion impermeable membrane.

pH titration of chloroplast suspensions

The effect of high concentration of sucrose on the internal pH of chloroplasts is also reflected in changes in buffer capacity. The acid–base titration curves are shifted considerably when the tonicity is shifted with either sucrose or NaCl (Fig. 8). At 1.0 M compared to 0.05 M NaCl, the chloroplasts require about four times more acid to drop from pH 9.0 to 7.0. Similar shifts were found by increasing the sucrose concentration. This result is consistent with the observation that the internal pH is higher at 1.0 than at 0.05 M NaCl. An alternative explanation for this phenomenon is that at high sucrose or salt concentration, polyelectrolytes tend to undergo an unwinding or extension due to the masking of the fixed charge groups resulting in a decrease in the electrostatic attraction. This could lead to the exposure of more dissociable groups which would then take part in the acid–base equilibrium, giving a higher buffer capacity.

Relationships of osmotically, pH-, and light-induced shrinkage

The most convenient method for measuring the light-induced shrinkage is the light-scattering procedure. Unfortunately, the method does not give absolute volumes. On the other hand, the chlorocrit method together with iodinated albumin to measure free space, as used in the present study, does give an absolute measure of volume. To make cross-comparisons, measurements of both light scattering and of packed volumes were made on chloroplasts exposed to different tonicity and pH's. The slope of the light scattering–volume relationship was greater for pH-induced changes (Fig. 9). In other words a decrease in volume induced by pH gave a greater change in light scattering than the same decrease in volume induced by a change in tonicity. Another difference was found when the range of volume changes was extended. Changes due to pH, especially at high volumes, were distinctly non-linear, whereas those due to tonicity were almost linear (Fig. 10). One difficulty in interpreting these data is the possible nullifying effect of the increase in sucrose concentration on the refractive index difference between the solution and the interior of the particles (since light-scattering intensity is directly proportional to the square of the difference in the refractive indices). Such an effect would be expected to decrease the slope of the osmotic plots given in Fig. 10, especially at high concentrations of sucrose. Yet at 0.133 M sucrose the curve generated by changing the pH is steeper than those at lower sucrose concentration. Therefore, it is concluded that changing the volume of plastids by changing tonicity results in a quantitatively different (and smaller) change in light-scattering intensity than would occur if that same volume change occurred due to changing the pH. This point becomes meaningful when one studies the capacity of the plastids used in the experiment of Fig. 9, to undergo the light-induced shrinkage. Two of the samples used in Fig. 9 were given 0.13 mM reduced trimethylbenzoquinone and exposed to red light, and their shrinkage was followed by light-scattering changes. The extent of the light-induced shrinkage is shown by the dashed right angle lines in Fig. 9. Both samples (a, 0.05 M sucrose, Tris acetate (pH 7.0); b, 0.1 M sucrose, Tris acetate (pH 6.0)) gave the same final level of light-scattering intensity. An inspection of the data shows that the increased



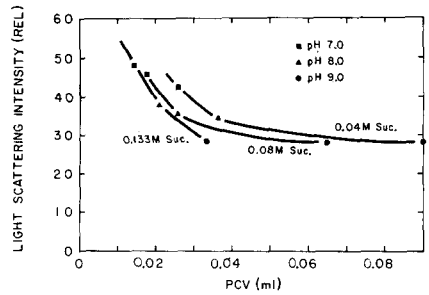


Fig. 9. Volume changes induced by pH and osmotic pressure. Chloroplasts were subjected to various pH values (at constant tonicity) and various tonicities (at constant pH). Light-scattering intensity and packed-cell volume were measured. For the pH-induced volume changes pH values of 6.0, 7.0, 8.0 and 9.0 in 0.05 M Tris acetate buffer were used, with sodium acetate added in the appropriate quantity to give equal tonicity. In addition to the salts mentioned, each sample taken for packed-cell volume measurement contained 0.1 M sucrose and 0.2 ml chloroplast suspension containing 2.0 mg/ml chlorophyll in a final volume of 0.6 ml. For the light-scattering measurements, 0.05 ml of chloroplasts were added to 3.0 ml of the stock sucrose and buffer solution. For the tonicity-induced volume changes, sucrose solutions of 0.06, 0.12, 0.15, and 0.2 M were used, with 0.05 M Tris acetate (pH 7.0), other conditions and additions were as outlined above. Two samples used to measure the light-scattering intensity (a, pH 6.0, 0.1 M sucrose; b, pH 7.0, 0.05 M sucrose) were given 0.13 mM reduced trimethylbenzoquinone and exposed to red light to measure the light-scattering change[5] which occurs during the light-induced shrinkage. These data were obtained with the instrument gain at the same value as used for the light-scattering measurements described above. The packed-cell volume (PCV) measurements were corrected for free space, using [^{131}I]albumin as the free space indicator.

Fig. 10. Light-scattering intensity *vs.* packed-cell volume (PCV) of plastids at three pH values and three sucrose (Suc.) tonicities. The packed-cell volumes were measured in 10 ml hematocrit tubes. The reaction mixtures contained in 15 ml total volume, the following: sucrose at 0.04, 0.08, or 0.133 M as indicated; Tris acetate buffer at pH 7.0, 8.0, or 9.0, as indicated; sodium acetate as required to maintain equimolar conditions at the three pH values; 0.9 mg chlorophyll equivalent chloroplasts. 10 ml of each sample was given 30 min of centrifugation to obtain the packed-cell volumes, 3 ml were used for light-scattering studies, and 2 ml for [^{131}I]albumin free space measurement.

light scattering can be accommodated only by the pH curve; the extrapolation to the osmotic curve would require the plastid volume to take on a negative value. The amount of light-induced shrinkage estimated from the pH curve is 67% for sample a and 57% for sample b. This amount of shrinkage is in agreement with that reported by ITOH, IZAWA AND SHIBATA[27] who measured light-induced shrinkage with the Coulter counter. The data suggest that the light-induced shrinkage occurs *via* a mechanism which is similar to that involved in pH-induced shrinkage, in terms of the quantitative changes in light-scattering properties. These data also indicate that at least in part, different substructures must be involved in the pH-induced and osmotically induced volume changes.

DISCUSSION

Spinach chloroplasts shrink and swell as reversible osmometers in response to changes in the tonicity of the suspending medium over a limited range of concentration, *i.e.*, 0.08 to 0.8 M sucrose or salt, in agreement with the results of TOLBERG AND MACEY[11]. Below 0.05 M sucrose the Boyle–Van 't Hoff law is still obeyed, but

the plot has a lesser slope which is interpreted as evidence that the osmotic compartment occupies a smaller volume, resulting perhaps from the swelling to the bursting point of some part of the internal structures. Alternatively, the membrane of part of the osmotic compartment which functions at higher tonicities becomes permeable in the concentration range of 0.05 M sucrose, leaving a smaller osmotically responsive volume. The observation of SPENCER AND WILDMAN[28] and MERCER *et al.*[15] show that around 0.05 to 0.01 M sucrose, extensive blebing of stroma lamellae occurs suggesting that the highly stretched membranes may become leaky or burst, or both. The swelling in dilute sucrose (16 mM) was only partially reversible, which could be due to a permanent distortion of the membrane during the blebing stage.

[^{131}I]Albumin provides a convenient means of defining the free space of a chloroplast pellet[29]. The average albumin space of a pellet is 45%, about the same as the dextran free space value for mitochondria pellets (43%) found by O'BRIEN AND BRIERLEY[30]. It is reasonable to define the albumin space as the space outside of the osmotic membrane of the particles. Using this criterion, a comparison of the distribution volumes of sucrose and albumin together with the analysis of the relative size of the osmotic compartment deduced from the *V vs.* $1/\Pi$ plots leads to the conclusion that the osmotic compartment (impermeable to sucrose and salts) constitutes about 25% of the plastid volume at 0.1 M sucrose. On the basis of this calculation it can be suggested that the osmotic compartment in question is the thylakoid (*i.e.*, the grana and stroma lamellae). Earlier work[13] has shown that isolated grana do exhibit osmotic behavior. Furthermore, ITOH, IZAWA AND SHIBATA[23] have shown that the total grana volume of spinach chloroplasts is about 30% of the chloroplast volume (in 0.35 M NaCl), which is consistent with our conclusion. The extrapolated *b* value (non-solvent volume) from our *V vs.* $1/\Pi$ plots (Figs. 1a and 1b) is about 8 μ^3 per chloroplast, which is close to the estimated total volume of grana in a spinach chloroplast[23]. This suggests that the packed-cell technique, using albumin to correct for free space, is measuring mostly the grana volume with at most a small contribution due to intact chloroplasts. At 0.1 M sucrose, the volume per chloroplast from Fig. 1a is 14 μ^3, a value similar to that found for chloroplasts in 0.1 M KCl using the Coulter counter technique[5]. In the latter case it was known that the plastid outer membranes were mostly ruptured, thus the Coulter counter was probably measuring the volume of the grana space.

The large degree of sucrose penetration compared to albumin is difficult to rationalize in view of the fact that these chloroplasts were osmotically responsive toward sucrose. Further work is required to elucidate this point. The model pre sented earlier[36] based on this work was probably not entirely correct.

Small ions and molecules such as Rb^+, Cl^-, and DMO distribute in a larger volume of the chloroplast than does sucrose (Tables I, III and IV).

The grana and stroma lamellae are pictured as osmotic compartments, impermeable to sucrose, permeable to undissociated DMO and to small anions (Cl^-), and permeable to cations only by a cation-exchange mechanism. It is necessary to impose the latter assumption in order to explain why a compartment which is permeable to individual ions responds osmotically to the salt concentration. A membrane need be impermeable to only one species of a salt to be effectively impermeable to the salt, for reasons of maintaining electric neutrality. For instance, the red blood cell is very permeably to anions but virtually impermeable to cations and effectively imper-

meable to the salt[31]. The same is true of many biological membranes. In such a situation the ion to which the membrane is relatively impermeable (*e.g.* K^+ in the red cell) may still be taken into the cell either by an exchange mechanism or by an active transport mechanism. It has been shown by TOLBERG AND MACEY[11] that K^+, Na^+, and Cl^- are not excluded from plastids, and in fact at low external Cl^-, there is a slight degree of concentration of Cl^- within the plastid, either as a counter-ion for K^+, or as a mobile anion for a Donnan system of fixed positive charges. Isolated chloroplasts of Nitella are more permeable to Cl^- than to Na^+ or K^+ (personal communication from Dr. PAUL SALTMAN). The penetration of cations into the grana by exchange has been demonstrated by studies of light-induced shrinkage. The grana is the primary unit that shrinks[13,18,32-34]. The shrinkage is accompanied by a net efflux of K^+ and Mg^{2+}, and a net influx of H^+, whereas the re-swelling in the dark is accompanied by an influx of K^+ and Mg^{2+}, and efflux of H^+ suggesting a cation-exchange system[4,5].

Because the components of the chloroplast structure contain charged groups, a Donnan component to the ion distributions in all compartments is to be expected. Furthermore, because many of the charged groups are dissociable at physiological pH, the internal pH of the compartments should greatly modify the Donnan effect. At pH values around neutrality most proteins and phophoryl compounds have a net negative charge. Indeed the ratio of inside to outside concentrations is higher for Rb^+ than for Cl^-, consistent with an average net negative charge in the chloroplast (Table II). It is also evident that Rb^+ and Cl^- do not respond to identical Donnan groups because the ratios are not reciprocally related (Table II). The Cl^- ratio should be considerably less than 1.0, but it is not. The surplus of Cl^- must be due to a compartment containing positive fixed charges, which is inaccessible to Rb^+. In the model it is proposed that this compartment is composed of the grana membranes themselves, with the positive groups within the membrane structure. Such positively charged membranes would account for the proposed anion permeability and cation impermeability. Evidence for positive fixed charge membranes in biological systems has been presented in the case of the red blood cell[35] accounting for the high permeability to anions and low permeability to cations. The positive charges, in this case, are probably free amino groups, based on studies of the effects of pH on permeability.

The importance of the Donnan effect imposed by dissociable fixed charges in the control of plastid volume is strongly suggested by the large volume changes that are produced by changes in pH, as demonstrated in the present and previous[15] studies. At the isoelectric point, the chloroplast is at minimum volume and it shows little response to the external osmotic pressure (Fig. 3), presumably because the concentration of net negative fixed charges in the osmotic compartment is markedly reduced, leading to a loss of counter-ions and of water. At reduced volume the compartment is such a small fraction of the total plastid volume that any changes in response to tonicity are minimal.

The interrelationship between fixed charges and chloroplast volume is also demonstrated by the changes in ion distribution and in internal pH when the chloroplasts are shrunken by high tonicity (0.5 to 0.8 M sucrose). The plastids lose H^+ and Rb^+ and gain Cl^- (Fig. 7 and Table V), suggesting a net decrease in the negative fixed charges. This result would be expected because a decreased volume would

result in an increased concentration of fixed charges, which in turn would lead to association with H^+, to a reduction in the number of fixed negative charges, and to a readjustment of the ion distribution.

The osmotically responsive compartment is not the only part of the chloroplast in which fixed charges are important in volume regulation. The osmotically unresponsive volume, represented by the intercepts of the Van 't Hoff plots (Figs. 2, 4 and 6) changes in volume with changes in pH, the presence of salts (*vs.* sucrose) and the presence of bivalent cations.

From the concentrations of Rb^+ inside the plastid and in the external solution one may calculate the concentration of the fixed anions $[A^-]$ by using the equation:

$$[Rb^+]_i = {}^1/_2 \{([A^-]^2 + 4\,[Rb^+]_0)^{\frac{1}{2}} - [A^-]\}$$

At 0.01 M Tris–HCl (pH 7.0), 0.1 M sucrose, approx. 1 mg chlorophyll per ml, and 0.01 M RbCl in the external solution, the average calculated $[A^-]$ from 4 experiments was 14 mM. This value was calculated by assuming 75% of the plastid volume is available to the Rb^+ through the K^+-exchange mechanism which occurs in chloroplasts[5]. The 75% water space value was obtained from gravinometric water analysis. The value of 14 mM for $[A^-]$ agrees quite well with the 20 mM value obtained by MERCER *et al.*[15] for Nitella chloroplasts.

The effect of Zn^{2+} (and certain other divalent cations, see Table III) on the volume of chloroplasts is similar to the effect of H^+, in that the plastids shrink to a volume minimum. The fact that Ca^{2+} and Mg^{2+} do not give the same result, and the influence of pH on the Mn^{2+}-induced shrinkage suggests that this type of shrinkage is due to an interaction of the effective ions with the ionized acidic groups and not just an ionic strength effect. The identity of the charged groups is not known but from the effect of pH on divalent cation-induced shrinkage and on the volume–osmotic pressure relationship it is apparent that groups which ionize in the pH range from 7.0 to 8.5 are involved in volume control as well as groups ionizing in the more acid range.

It seems evident from the comparison of the light-scattering changes brought about by the three types of shrinkage, that the light-induced shrinkage more closely resembles the pH rather than the osmotic pressure-induced shrinkage. This point is further corroborated by the effect of divalent cations such as Zn^{2+}, Mn^{2+}, *etc.*, in which a contraction of the plastid by the Zn^{2+} mimicks the effect of pH 4.7 treatment. It has been well documented[26] that ions such as Zn^{2+} and Cu^{2+} compete with H^+ for imadazolium binding sites of protein such as metmyoglobin, and that such metal binding results in much the same conformational changes as those induced by acid treatment.

Further evidence linking the type of changes generated by the light-induced shrinkage with those caused by a pH decrease is to be found in the light-induced proton uptake[3,5]. It has been clearly demonstrated that light-induced chloroplast shrinkage is preceded slightly by an uptake of protons and an efflux of K^+ and Mg^{2+}. It has been shown here by the data on Rb^+ distribution that during a decrease in pH from 7.0 to 4.7 a net Rb^+ efflux from the chloroplast is observed while fixed acid groups become protonated, and the plastid shrinks. These considerations make very plausible the conclusion that the light-induced shrinkage is driven by a mechanism which is very similar to the pH-induced shrinkage.

DILLEY AND VERNON[5] postulated a model system in which the reversible protonation of fixed negative charge groups in the plastid grana system is a primary event leading to the light-induced loss of cations and water. The present work indicates that the fixed charge groups which are present in plastids can have a significant influence on the control of plastid volume and cation content in a passive sense (*i.e.* apart from functional reactions). This would appear to be a necessary (but not sufficient) condition for fixed charge groups to fulfil to be consistent with the hypothesis. Experiments designed to identify and characterize the fixed groups which are alluded to in this report may be an important part of gaining understanding of the mechanism of ion transport in chloroplasts.

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