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ELECTROLYTE EXCHANGE IN ISOLATED SPINACH CHLOROPLASTS

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SUMMARY

1. Efflux of $^{22}Na^+$, $^{42}K^+$, and $^{36}Cl^-$ from isolated chloroplasts was measured in the relative absence of metabolic activity, *i.e.*, in the dark at 4°. The half-time for isotope efflux was approx. 11 min for all 3 ions.

2. The size of the Na^+ pool was proportional to the Na^+ concentration in the medium; Na^+ efflux was proportional to the size of the pool.

3. When chloroplasts were disrupted by alternate freezing and thawing, Na^+ efflux kinetics were not altered. However, the exchanging pool size was reduced about 70 % in the disrupted chloroplasts.

4. At 24° the efflux half-time decreased to 4 min. This corresponds to an activation energy of 7650 cal/degree per mole.

5. On the basis of these observations it is suggested that the exchanging compartment corresponds to small subcompartments within the chloroplast, and comprises less than 10 % of the pellet volume. The ions appear to penetrate the diffusion barriers by simple diffusion. The diffusion barriers separating the small subcompartments from the external compartment appear to be highly permeable to ions, and there is no evidence of a Donnan distribution or of a transmembrane potential.

INTRODUCTION

In spite of the current interest in light-induced ion uptake, the passive transport of small inorganic ions in chloroplasts has been sparsely studied. Much of what is known has been inferred from osmotic behavior and from limited chemical and isotope tracer studies, and there are a number of inconsistencies among the data reported. These studies were undertaken in an effort to elucidate the passive permeability of chloroplasts to Na^+, K^+ and Cl^-.

A relative impermeability to various solutes has been suggested from osmotic studies of spinach chloroplasts[1,2] and algal chloroplasts isolated from Nitella[3]. Chloroplast volume changes occurred in accordance with the Boyle–Van't Hoff relation. Isotope exchange studies with Nitella chloroplasts[4] are consistent in demonstrating that K^+ equilibrates only slowly between chloroplasts and medium, while Na^+ appears to be excluded from the chloroplasts.

On the other hand, chemical analyses of spinach chloroplasts (isolated under a variety of conditions) imply that small inorganic ions pass easily between chloroplasts and the medium. TOLBERG AND MACEY[2] reported that chloroplast pellet Na^+,

K^+ and Cl^- concentrations resembled closely those of the high salt medium employed in isolation procedures. There is also a substantial leaching of inorganic ions from chloroplasts isolated in sucrose[2,5]. A single washing with sucrose resulted in a loss of 80 % of the K^+ and Na^+ found in sucrose-isolated plastids[6].

In many instances changes of chloroplast volume have been interpreted as an osmotic response to solute penetration, *e.g.*, the slow swelling of chloroplasts in the dark[5,7,8] and under normal room illumination[1]. The permeability of spinach chloroplasts to weak acids and ammonia has been inferred from the rate and extent of chloroplast swelling[5].

Because of the apparent inconsistencies between data on chloroplast volume changes and chemical analyses, and because of the extension of these methods to study effects of light and metabolism on ion uptake, it seemed desirable to examine more critically and directly the passive ion permeability of isolated spinach chloroplasts. We have chosen to examine the kinetics of exchange of Na^+, K^+, and Cl^- between chloroplasts and the suspending medium under conditions of minimal metabolic activity, *i.e.*, in the dark and in the cold.

MATERIALS AND METHODS

Chloroplasts were isolated directly from juice extracted from spinach leaves, as described previously[2]. Briefly, the juice obtained from spinach leaves by a juice extractor was strained through gauze, and chloroplasts isolated by differential centrifugation. The supernatant juice was then centrifuged at 32000 $\times$ *g* for 20 min to remove most of the remaining particulate debris and was used as suspending medium in some experiments.

The isolated chloroplast pellet was gently resuspended in approx. 5 ml of chilled medium which contained 10^5 to 10^6 counts/min per ml of the radioactive isotope(s). When juice was not used, the salt solutions were buffered to pH 6.5 with phosphate buffer. The isotope was allowed to equilibrate with the suspended chloroplasts for about 60 min. The suspension was then centrifuged at 1000 $\times$ *g* for 10 min and the radioactive supernatant removed as completely as possible. A small aliquot of this radioactive supernatant was counted for isotope content, referred to as Y_{mo}^*. At the start of the efflux period, the chloroplast material equilibrated with isotope (about 1.5 ml) was rapidly resuspended in 15 to 20 ml of isotope-free medium, otherwise identical in composition to that used for incubation. After periods of 1 to 60 min, 0.8-ml aliquots of this mixture were removed and centrifuged for 30 to 60 sec at about 29000 $\times$ *g* in a Misco Model 5500 microcentrifuge (Microchemical Specialties Co., Berkeley, Calif.). This centrifuge reaches maximum speed within 5–10 sec and permits rapid sampling of small volumes. The centrifuge was rapidly braked, the supernatant poured off, and the surface of the pellets rinsed quickly 3 times with the same medium containing no isotope. Packing was sufficiently tight that no perceptible amount of pellet was lost in the rinses. Pellets were counted for isotope content. At the conclusion of the experiments an aliquot of the efflux reaction mixture (chloroplasts and medium) was assayed for radioactivity and for chlorophyll content.

As a control, $^{22}Na^+$ efflux from chloroplasts was compared with that from a pellet of polystyrene spheres (diameter, 12 μ). The spheres were presumably impermeable to the isotope and would be expected to give a constant level of radioactivity

in an efflux experiment. The plot in Fig. 1 conforms to that predicted and indicates that the kinetics of sodium efflux from chloroplasts represents a property of the chloroplasts and is not an artifact of experimental procedure.

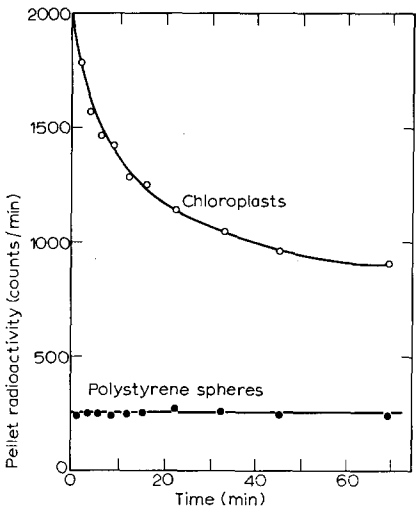

Fig. 1. Comparison of $^{22}Na^+$ efflux from polystyrene spheres and chloroplasts. Pellet counts (Y_p^*) are plotted as function of time.

Doubly labelled experiments were run in which efflux of $^{42}K^+$ was compared to either $^{22}Na^+$ or $^{36}Cl^-$ efflux in the same chloroplast preparation. Sodium or chloride isotope was added to medium already containing $^{42}K^+$. Assays of radioactivity were made upon completion of the experiment and again one week later, at which time the $^{42}K^+$ had decayed to background levels. The difference between initial and final counts was considered to be the $^{42}K^+$. These values were appropriately corrected for decay which occurred during the counting procedure.

In order to compare efflux from intact chloroplasts with that from disrupted chloroplasts, the chloroplast pellet was alternately frozen, by dipping the test tube into isopropanol–dry ice mixture, and thawed, by immersing the tube in warm water. The pellet was stirred with a glass rod to hasten thawing. This procedure was repeated 3 times, taking a total of about 3 min. Isotope efflux was followed by resuspending the disrupted pellet and an intact control pellet simultaneously in identical volumes of efflux medium.

Packed chloroplast volumes were measured in the cold by microhematocrit[2]. Chlorophyll content of the chloroplast suspensions was determined by the method of ARNON[9]. Na^+ and K^+ contents of juice were determined by flame photometry of trichloroacetic acid extracts. Chloride determinations were made with a Cotlove chloridometer, and osmotic pressures of suspending media were determined with a Fiske osmometer.

CALCULATIONS

Data could be interpreted in terms of a single compartment model. For this analysis Na^+, K^+, and Cl^- were assumed to reside within single compartment pool(s).

The mechanism of exchange between a given pool and the bathing medium is not specified.

Let Y_c be the amount of any particular solute species (*e.g.* Na^+) in the chloroplast pool at any time t, and Y_c^* be the corresponding amount of tracer in the pool. Similarly, let Y_m and Y_m^* represent the concentrations of the solute and of tracer solute, respectively, in the medium at time t. Let J denote the steady-state flux of the solute in question. Then the rate of change of tracer within the chloroplast, dY_c^*/dt can be described by Eqn. 1.

$$\frac{dY_c^*}{dt} = J\left[\frac{Y_m^*}{Y_m} - \frac{Y_c^*}{Y_c}\right] \tag{1}$$

In the steady state J, Y_m and Y_c are independent of time. Since the volume of medium is very large compared to the chloroplast pool, Y_m^* is essentially constant. If we let Y_{co}^* denote Y_c^* at time $t = 0$, the integral of Eqn. 1 becomes

$$Y_c^* = \frac{Y_m^*}{Y_m} \cdot Y_c + \left[Y_{co}^* - \frac{Y_m^*}{Y_m} \cdot Y_c\right] \exp\left(-\frac{J}{Y_c} \cdot t\right) \tag{2}$$

When t becomes large Y_c^* equilibrates with the medium and approaches a constant asymptotic value, $Y_{c\infty}^*$. From Eqn. 2 it follows that $Y_{c\infty}^* = (Y_m^*/Y_m)Y_c$ so that Eqn. 2 can be rewritten as

$$Y_c^* = Y_{c\infty}^* + (Y_{co}^* - Y_{c\infty}^*) \exp\left(-\frac{J}{Y_c} \cdot t\right) \tag{3}$$

Eqn. 3 is not sufficient for the interpretation of data because experimental measurements were made on centrifuged pellets which consist of the exchanging solute pool compartment (Y_c) *plus* another compartment which is contiguous with and essentially in equilibrium with the suspending medium. Let Y_p and Y_p^* denote the amount of the particular solute and tracer, respectively, in the pellet at time t, and Y_e and Y_e^* the portion of pellet solute and tracer, respectively, which are in equilibrium with the medium, and hence remain constant throughout the efflux experiment.
Then

$$Y_p^* = Y_c^* + Y_e^* \tag{4}$$

and Eqn. 3 can be rewritten in terms of pellet counts obtained experimentally:

$$Y_p^* = Y_{p\infty}^* + (Y_{po}^* - Y_{p\infty}^*) \exp\left(-\frac{J}{Y_c} \cdot t\right) \tag{5}$$

A numerical procedure (least squares) was used to determine the three parameters of Eqn. 3, *i.e.*, the decay constant J/Y_c, the asymptote $Y_{p\infty}^*$, and the coefficient $(Y_{po}^* - Y_{p\infty}^*)$. The efflux half-time, $t_{1/2}$, was calculated from $t_{1/2} = 0.69\ Y_c/J$, and the flux, J, can be calculated from the decay constant, J/Y_c, once Y_c is known.

To estimate Y_c, let Y_{mo}^* represent the concentration of tracer solute in the incubation medium from which the chloroplasts were separated just prior to the efflux experiment. If equilibration is complete, the specific activities should be identical in the chloroplast pool and in the medium, and

$$Y_{co}^* = Y_{mo}^* \cdot \frac{Y_c}{Y_m} \tag{6a}$$

Similarly, a new equilibrium occurs following the efflux experiments, when $t = \infty$, so that

$$Y_{c\infty}{}^{*} = Y_{m}{}^{*} \frac{Y_c}{Y_m} \tag{6b}$$

Using the relations $Y_{po}{}^{*} = Y_{co}{}^{*} + Y_e{}^{*}$ and $Y_{p\infty}{}^{*} = Y_{c\infty}{}^{*} + Y_e{}^{*}$, $Y_{co}{}^{*}$ and $Y_{c\infty}{}^{*}$ can be eliminated from Eqns. 6a and 6b to give

$$\frac{Y_c}{Y_m} = \frac{Y_{po}{}^{*} - Y_{p\infty}{}^{*}}{Y_{mo}{}^{*} - Y_{m}{}^{*}} \tag{7}$$

RESULTS

Efflux of $^{22}Na^{+}$, $^{42}K^{+}$, and $^{36}Cl^{-}$

Figs. 1 and 2 illustrate the results of typical efflux experiments. Efflux of isotopic Na^{+}, K^{+} and Cl^{-} all occurred in the exponential fashion predicted by Eqn. 5.

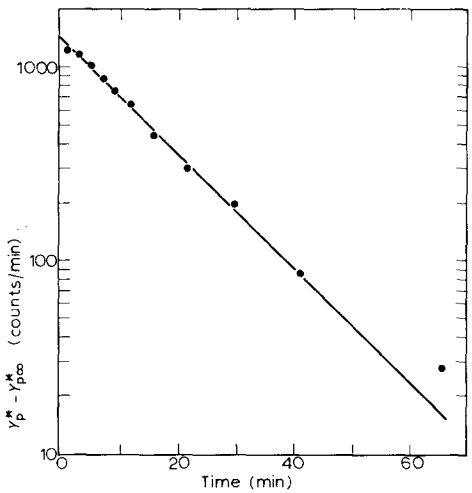

Fig. 2. Typical plot of $^{22}Na^{+}$ efflux from isolated spinach chloroplasts. Data are plotted with difference between total and final pellet counts ($Y_p{}^{*} - Y_{p\infty}{}^{*}$) on the ordinate (note logarithmic scale) and with time on the abscissa.

The time course was the same for Na^{+}, K^{+} and Cl^{-} (see Table I). Assuming efflux and influx rates are of the same order, the efflux half-time of 10 min implies that equilibration of the isotopes was essentially complete at the end of the pre-efflux incubation period (usually 1 h) and at the end of the efflux run (60 to 90 min).

In Table I, pool sizes Y_c were divided by medium concentration Y_m to facilitate comparisons when medium ion concentrations differ. This gives an estimate of the compartment volume per mg chlorophyll. The results show that the volume of distribution of each ion within the compartment is similar.

Dependence of Na^{+} efflux on Na^{+} concentration

Data from efflux of $^{22}Na^{+}$ into media of different sodium concentrations is shown in Table II. Estimated chloroplast pool sizes Y_c were approximately pro-

portional to concentrations. On the basis of these pool sizes, Na^+ fluxes may be calculated from the decay constant, J/Y_c. It was found that fluxes were proportinal to pool size, implying that efflux half-times are independent of pool size and hence of medium concentration. The mean values for efflux half-times were found to be similar over Na^+ concentrations of less than 1 to 350 mM. (See Table II.)

Efflux from disrupted chloroplasts

If the barrier to diffusion of ions out of chloroplasts is due to the presence of a single chloroplast compartment separated from the efflux medium by a simple membrane, the efflux should be considerably more rapid if the membrane is damaged. Following incubation in the presence of $^{22}Na^+$, chloroplasts were alternately frozen and thawed in order to disrupt any such membranes, and the efflux of $^{22}Na^+$ was followed as in previous experiments. The results are summarized in Table III. The efflux half-time was not altered by disruption. However, from the values of

TABLE I

COMPARISONS OF EFFLUX HALF-TIMES FOR $^{22}Na^+$, $^{42}K^+$, AND $^{36}Cl^-$, AND OF ISOTOPE DISTRIBUTIONS IN THE EXCHANGING POOLS (Y_c)

Half-times were determined in a variety of media, including salt solutions of various concentrations and juice. The data were pooled since there was no evidence that the half-time differed in the various media used. Pool sizes were obtained using efflux medium containing 190 mM Na^+, 190 mM K^+, 350 mM Cl^-, and 20 mM phosphate buffer (pH 6.5). Data are expressed as mean ± standard error of the mean. The figure in parentheses refers to the number of experiments included in the data.

Isotope	*Half-time (min)*	Y_c/Y_m *(μl/mg chlorophyll)*
$^{22}Na^+$	10.7 ± 0.6 (55)	2.60 ± 0.06 (4)*
$^{42}K^+$	11.9 ± 1.1 (13)	2.47 ± 0.21 (8)
$^{36}Cl^-$	10.3 ± 1.5 (10)	2.53 ± 0.11 (4)

* When values of Y_c/Y_m for Na^+ and K^+ were estimated on the same pellets, they were found to be 2.60 and 2.15 μl/mg chlorophyll, respectively. The 20% difference of 0.45 ± 0.12 μl/mg chlorophyll was significant ($P < 0.01$).

TABLE II

EFFECT OF Na^+ CONCENTRATION ON $^{22}Na^+$ EFFLUX

Chloroplasts were washed and incubated with isotope in the various efflux media. In media containing less than 0.35 M NaCl, salt concentration was maintained by substituting choline chloride. Media were buffered to pH 6.5 with 20 mM potassium phosphate. Data are means of 3 experiments.

Na^+ concn. in medium (Y_m) (mequiv/l)	*Na^+ pool (Y_c) (10^2 × μmoles per mg chlorophyll)*	*Na^+ flux (J) (10^3 × μmoles per mg chlorophyll/min)*	J/Y_c *(10^2 × min^{-1})*	*Half-time (min)*	Y_c/Y_m *(μl/mg chlorophylll)*
<1	<0.42	<0.21	5.0	13.6 ± 2.8*	4.2 ± 0.7
7.5	2.4	1.37	5.7	13.2 ± 2.5	3.2 ± 0.5
75	31	14.2	4.6	14.9 ± 2.1	4.2 ± 0.8
350	114	55	4.8	14.4 ± 1.0	3.2 ± 0.1

* Mean ± S.E.

($Y_{p0}^* - Y_{p\infty}^*$) we can infer that the exchanging pool was reduced by over 70 % in the disrupted chloroplasts.

TABLE III

EFFECT OF ALTERNATE FREEZING AND THAWING ON ^{22}Na EFFLUX FROM ISOLATED SPINACH CHLOROPLASTS

Chloroplasts were suspended in spinach juice or in 0.35 M NaCl buffered to pH 6.5 with 20 mM potassium phosphate buffer. ($Y_{p0}^* - Y_{p\infty}^*$) normalized to counts/min mg chlorophyll was used to estimate the relative size of the exchanging pools. Data are expressed as mean ± S.E. Figure in parenthesis refers to number of experiments included in the data.

Chloroplasts	*Half-time (min)*	$Y_{p0}^* - Y_{p\infty}^*$ *(% of control value)*
Intact	9.2 ± 0.8 (10)	100 (8)
Disrupted	10.4 ± 1.3 (10)	27 ± 4 (8)

Effect of temperature on sodium efflux

The relatively rapid efflux at low temperature suggested that the energy barriers to ion efflux from chloroplasts are low, and might resemble those of diffusion in water rather than transport through cell membranes. Measurement of Na^+ efflux at room temperature was also of interest since many studies of chloroplast function are made under these conditions. The 20° increase in temperature from 4° to 24° decreased the Na^+ efflux half-time from 11.3 to 4.0 min. This corresponds to an activation energy of 7650 cal/degree per mole. This value is slightly higher than the value for free diffusion in water but considerably lower than those for diffusion through cell membranes.

DISCUSSION

Since these experiments were performed in the relative absence of metabolic activity (in the dark at low temperature), it can be assumed that mainly passive transport mechanisms are involved in the ion efflux, *i.e.*, simple diffusion, exchange diffusion, or facilitated diffusion.

The barrier to ion diffusion in chloroplasts appears to be quite loose as compared to cell membranes. This can be inferred from the low energy of activation and from the apparent lack of specificity, *i.e.*, from the similarity of rapid efflux kinetics and from the similarity of pool sizes for Na^+, K^+, and Cl^-. Further, assuming that the ions are distributed within the same compartment, the fact that Y_c/Y_m does not differ for anions and cations implies that this ion distribution is not affected by an electrical potential difference across the exchange barrier. In other words the net fixed charge concentration of any impermeable ions within the compartment is insufficient to cause an imbalance of permeable ion concentrations, and the Donnan ratio is close to unity. This observation appears to be inconsistent with the report of DILLEY AND ROTHSTEIN[10] that the $^{86}Rb^+$ distribution ratio between chloroplasts and medium was 1.6. The apparent discrepancy may result from the fact that their experiments were performed at much lower salt concentrations than those used in our experiments.

In the absence of a membrane potential, efflux of Na^+ by simple diffusion should

be proportional to pool size. The data in Table II show that this is the case. Further, there is no evidence for exchange diffusion. Efflux of Na^+ increases with an increase of Na^+ concentration in the medium, but this is quantitatively accounted for by the corresponding increase in the compartmental Na^+ pool. Thus the exponential decay constant is given by J/Y_c, and if, as we have seen, J is proportional to Y_c, then the decay constant should remain invariant when Y_c (or Y_m) is changed. The values of J/Y_c and half-times in Table II show this to be the case.

If it is true that the ions passively diffuse and equilibrate across the chloroplast membranes and that the membrane potential plays an insignificant role, then it would be expected that the ion concentrations would be uniformly distributed. Assuming a uniform distribution, the pellet volume can be estimated from $Y_{p_\infty}{}^*/Y_m{}^*$ and is found to be about 50 μl per mg chlorophyll. This value is in good agreement with 67 μl per mg chlorophyll for the packed volume of chloroplasts in 0.35 M NaCl (*cf.* TOLBERG AND MACEY[2]). (The packed volume figure includes 14 mg dry matter per mg chlorophyll.) A similar calculation of the compartment volume (Table I) reveals that Y_c/Y_m is of the order of 2.5 μl/mg chlorophyll. This indicates that the compartment is small relative to the volume of the chloroplasts and that the unit compartment corresponds to only a portion of the chloroplast particle. If the lamellar structure of the chloroplast is considered, it seems quite possible that each chloroplast might contain a number of similar compartments, separated from one another and from the medium by membranes, such as the stromal or granal lamellae. Chloroplast fragments, presumed to be stacks of grana, have been shown to undergo osmotically induced volume changes[11]. Such subcompartments might correspond to the isotope-retaining compartments and might be responsible for the osmotic volume changes of intact chloroplasts. Boyle–Van't Hoff plots of chloroplasts in media of varying osmotic concentrations show a large non-osmotic volume[2]. If Y_c/Y_m represents that portion of the chloroplast pellet which shows osmotic behavior, only a small proportion of the pellet would be osmotically active. Recent studies of DILLEY AND ROTHSTEIN[10] on osmotic behavior and sucrose distribution in chloroplasts support the hypothesis that the osmotically active compartment corresponds to the grana of the chloroplast.

When values of Y_c/Y_m for Na^+ and K^+ were estimated on the same pellets, the value for Na^+ was about 20 % higher than that for K^+. (See footnote in Table I.) While this may reflect some selectivity for Na^+ over K^+ within the exchanging compartment, it is relatively small, and cannot be accounted for on the basis of data obtained thus far. It is of some interest, however, in view of the report of enhanced uptake of Na^+ but not of K^+ by illuminated chloroplasts[12].

These studies of Na^+, K^+ and Cl^- efflux from isolated spinach chloroplasts indicate that the exchanging compartment represents less than 10 % of the pellet volume. It is suggested that the compartment may correspond to small subcompartments within the chloroplast. The diffusion barriers separating the compartment(s) from the medium appear to be extremely permeable, and the ions penetrate by simple diffusion. There is no evidence of a Donnan distribution or of a transmembrane potential.

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