*Biochimica et Biophysica Acta*, 637 (1981) 252–263
Elsevier/North-Holland Biomedical Press

BBA 48115

# THE CONCENTRATIONS AND THERMODYNAMIC ACTIVITIES OF CATIONS IN INTACT *BRYOPSIS* CHLOROPLASTS

AKIHIKO YAMAGISHI, KAZUHIKO SATOH and SAKAE KATOH

*Department of Pure and Applied Sciences, College of General Education, University of Tokyo, Tokyo 153 (Japan)*

(Received February 24th, 1981)

*Key words: Cation concentration; Ionophore; Thermodynamic activity; Internal pH; (Bryopsis chloroplast)*

**The internal cation levels of chloroplasts isolated from a green sea alga, *Bryopsis maxima*, were studied. Atomic absorption spectroscopy, combined with the determination of the sorbitol-impermeable and water-permeable spaces, revealed that chloroplasts contain an extremely high concentration of $K^+$ and high levels of $Na^+$, $Mg^{2+}$ and $Ca^{2+}$. A method was developed to estimate the thermodynamic activities of monovalent and divalent cations present in chloroplasts. pH changes induced by the addition of an ionophore (plus an $H^+$ carrier), which makes the outer limiting membranes of chloroplasts permeable to both a cation and $H^+$, were determined. Provided that the external pH was set equal to the internal pH, the internal concentration of the cation was estimated by determining the external cation concentration which gave rise to no electrochemical potential difference of the cation and hence no pH change on addition of the ionophore. The internal pH was determined by measuring distributions of radioactive methylamine and 5,5-dimethyloxazolidine-2,4-dione between the chloroplast and medium (Heldt, H.W., Werdan, K., Milovancev, M. and Geller, G. (1973) Biochim. Biophys. Acta 314, 224–241). The internal pH was also estimated by measuring pH changes caused by the disruption of the outer limiting membrane with Triton X-100. The results indicate that a significant part of the monovalent cations and most of the divalent cations are attracted into a diffuse layer adjacent to the negatively charged surfaces of membranes and proteins, or form complexes with organic and inorganic compounds present in the intact chloroplasts.**

## Introduction

Accumulating evidence of regulatory effects of monovalent and divalent cations on photochemical processes associated with the thylakoid membrane, such as chlorophyll *a* fluorescence [1–5], electron transport [6,7] and $H^+$ pumping [8,9], as well as on the functioning of $CO_2$-reducing enzymes in the stroma [10,11], emphasizes the need for information about the cation environment inside intact chloroplasts. However, the present knowledge of the internal levels of cations in chloroplasts is still unsatisfactory. The internal concentrations of several cations reported in the literature varied markedly depending upon the source and quality of intact chloroplasts tested and the methods used for determination of the intrachloroplast space [12–19]. Furthermore, no attempt has been made to determine cation levels in terms of the thermodynamic activities of cations, which from the physiological point of view are more important than their total concentrations. The effective concentrations of cations should be much lower than their total concentrations in intact chloroplasts which contain membranes and proteins with negative surface charges [3,19,20].

The aim of the present work was to determine the

---

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMO, 5,5-dimethyloxazolidine-2,4-dione; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; Chl, chlorophyll.

0005-2728/81/0000–0000/$02.50 © 1981 Elsevier/North-Holland Biomedical Press

internal cation levels of intact chloroplasts isolated from a marine green alga, *Bryopsis maxima*. The total concentrations of $K^+$, $Na^+$, $Mg^{2+}$ and $Ca^{2+}$ were estimated from their contents and the sorbitol-impermeable and water-permeable spaces of chloroplasts. In addition, a method was developed for the estimation of the thermodynamic activities of cations inside intact chloroplasts. The method consists of a series of pH measurements of chloroplast suspensions to which an ionophore and an $H^+$ carrier are added. For instance, on addition of valinomycin, an ionophore specific for $K^+$, and of CCCP, an $H^+$ carrier, $K^+$ and $H^+$ will be transported across the outer limiting membrane according to the electrochemical potential difference of $K^+$ ($\Delta\bar{\mu}_{K^+}$) and $H^+$ ($\Delta\bar{\mu}_{H^+}$) between the intrachloroplast space and the suspending medium, respectively.

$$\Delta\bar{\mu}_{K^+} = RT\ln([K^+]_o/[K^+]_i) + F\Delta\psi \qquad (1)$$

$$\Delta\bar{\mu}_{H^+} = RT\ln([H^+]_o/[H^+]_i) + F\Delta\psi$$

$$= 2.3RT\Delta\mathrm{pH} + F\Delta\psi \qquad (2)$$

where $\Delta\psi$ and $\Delta$pH are the electrical potential difference and pH difference across the outer limiting membrane, respectively. $[K^+]_i$ and $[K^+]_o$ are the internal and external activities of $K^+$, and $[H^+]_i$ and $[H^+]_o$ are the internal and external activities of $H^+$, respectively. $R$, $T$ and $F$ have their usual meanings. At equilibrium:

$$\Delta\bar{\mu}_{H^+} = \Delta\bar{\mu}_{K^+} = 0 \qquad (3)$$

We obtain:

$$[H^+]_i/[H^+]_o = [K^+]_i/[K^+]_o \qquad (4)$$

Eqn. 4 shows that $[K^+]_i$ can be estimated when $[H^+]_i$ is known. Especially when $[H^+]_o$ is equal to $[H^+]_i$, pH changes depend solely upon the ratio of $[K^+]_i$ to $[K^+]_o$. Thus, one can estimate $[K^+]_i$ by determining $[K^+]_o$ where no pH change will be induced by the addition of valinomycin and CCCP. In practice, the internal concentration of $K^+$ is estimated from the external $K^+$ concentration where no net $H^+$ transport across the outer limiting membrane occurs on simultaneous addition of valinomycin and CCCP. The $K^+$ concentration thus estimated is different from the total $K^+$ concentration inside chloroplasts as described in the text and hence is called the effective internal concentration of $K^+$. The thermodynamic activity of $K^+$ is calculated from its effective concentration using the activity coefficient given in the literature.

Other ionophores, such as nigericin or A23187, which facilitate $H^+$-cation exchange across the membrane, can be used to determine the internal activities of $K^+$ or divalent cations. In this case, $\Delta\bar{\mu}_{H^+} = \Delta\bar{\mu}_{K^+}$, or $2\Delta\bar{\mu}_{H^+} = \Delta\bar{\mu}_{\mathrm{divalent\,cation}}$, is not necessarily zero but Eqn. 4 or:

$$([H^+]_i/[H^+]_o)^2 = [M^{2+}]_i/[M^{2+}]_o \qquad (5)$$

still holds.

The most critical requirement for this method is to determine the internal pH ($pH_i$) accurately. $pH_i$ was estimated by measuring the distribution of radioactive methylamine and DMO between the medium and the intrachloroplast space according to the procedure of Heldt et al. [21]. An alternative method, in which $pH_i$ was determined by measuring pH changes caused by the disruption of the outer limiting membrane of chloroplasts with a nonionic detergent, was also employed.

## Materials and Methods

### *Chloroplast preparations*

*Bryopsis maxima* was collected at a seacoast near Choshi, Chiba Prefecture, and kept in cold seawater (approx. 6°C) in the dark. The alga was transferred to fresh seawater at 14°C and kept under a light(10 h)-dark(14 h) cycle for 2 days before use.

The long multinuclear cells were submerged in a preparation medium, cut into pieces and their contents were squeezed out through 16 layers of gauze. The medium was a modified solution B of Jensen and Bassham [22] containing 1.0 M sorbitol, 50 mM Hepes, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 2 mM EDTA, and 2 mM $NaNO_3$ (pH 6.7). The cell extracts were centrifuged at $1\,000 \times g$ for 90 s and the precipitates were suspended in 1.1 M sorbitol, the pH of which was adjusted to 7.5 with Tris base, followed by centrifugation at $100 \times g$ for 20 s to remove large debris and aggregates. Chloroplasts were collected by centrifugation at $1\,000 \times g$ for 90 s and washed once with

1.1 M sorbitol. It is important to remove the upper loose layer of the precipitate together with the supernatant after the final centrifugation to increase the population of intact chloroplasts. Microscopic inspection revealed that chloroplasts thus prepared were mostly intact and contamination by other cellular organelles was very small. The intactness of chloroplasts was also examined by the ferricyanide method [23]. Ferricyanide photoreduction was determined spectrophotometrically at 420 nm before and after the osmotic disruption of chloroplasts in 1 mM $MgCl_2$. The sample was illuminated with red light-(650–750 nm) of 44 $mW/cm^2$. The composition of the reaction medium was the same as that of the preparation medium except that 1 mM ferricyanide and 20 mM $NH_4Cl$ were added as an electron acceptor and an uncoupler, respectively, and the pH was adjusted to 7.3. The intactness of chloroplasts examined by this method was between 87 and 95%.

*Determination of pH changes*

The pH of chloroplast suspensions was monitored with a Horiba 6028 combination glass electrode connected with a Hitachi-Horiba F-7ss pH-meter. Chloroplasts equivalent to 100–200 $\mu$g Chl/ml were suspended in a medium (1.8 ml) containing 0.5 mM Hepes and 0.5 mM Mes. The medium also contained various concentrations of salts but its osmolality was maintained at 1.3 osmol/kg water by varying the sorbitol concentration. The initial pH of chloroplast suspensions as well as that of additions was adjusted to the desired values with NaOH or HCl. The amounts of $H^+$ appeared or disappeared and the buffering capacity of the suspension were titrated with 0.01 M HCl. All measurements were carried out at 10°C in the dark with continuous stirring.

*Determination of the internal volume and pH of intact chloroplasts*

The volume of the sorbitol-impermeable and water-permeable spaces of chloroplasts was determined by the method of Heldt and Sauer [24]. Chloroplasts were precipitated with a Beckman microfuge through a silicone layer (1 : 1 mixture of HIVAK F-4 and F-5, Shinetsu-Kagaku, $\rho = 1.075$). [$^{14}$C]Methylamine and [$^{14}$C]DMO were used to determine the pH in the stroma and intrathylakoid spaces according to the method of Heldt et al. [21]. The thylakoid space was assumed to be 12.5% of the total osmotic space of chloroplasts [21].

Radioactivity was determined with a Beckman LS 9000 liquid scintillation system using a xylene/Triton-type scintillation cocktail (Dojindo Labo. Scintisol-500).

## Results

### *Cation levels in intact chloroplasts*

*Dependence of photosynthetic $O_2$ evolution on medium osmolarity.* Photosynthetic $O_2$ evolution in intact chloroplasts of *B. maxima* depends strongly upon the sorbitol concentration of the suspending medium and shows maximum activity at about 1.0 M sorbitol (Fig. 1). Inactivation of the $O_2$ evolution at low sorbitol concentrations was accompanied by loss of the intactness of chloroplasts. Sorbitol can be replaced by lower concentrations of KCl. The optimum concentration of the salt was 0.6 M. However, when plotted on the osmolarity scale, the two curves

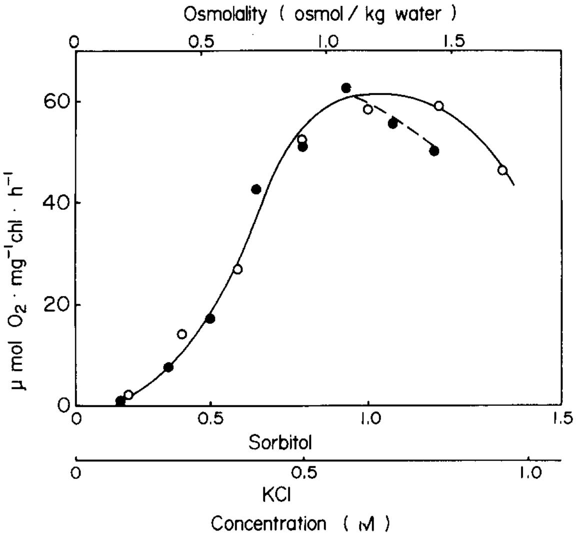

Fig. 1. Dependence of photosynthetic $O_2$ evolution on the medium osmolality. $O_2$ evolution was measured with a Clark-type oxygen electrode at 25°C. The reaction mixture contained 50 mM Tricine, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 5 mM $NaHCO_3$ and the indicated amounts of KCl (●) or sorbitol (○). The pH of the medium was adjusted to 8.1 with NaOH. The chloroplast concentration was equivalent to 47 $\mu$g Chl/ml. The samples was illuminated by white light (65 $mW/cm^2$).

coincided well with each other, except at superoptimum concentrations where the activity decreased more sharply in the presence of KCl than in the presence of sorbitol. The maximum activity was attained at 1.1–1.3 osmol/kg water with the two substances. The requirement of high osmolarity for the optimal functioning of photosynthesis has been described with chloroplasts isolated from other marine siphonales [25].

*Total cation concentrations.* The results above suggest that *Bryopsis* chloroplasts contain high concentrations of salts. This was confirmed by measuring the internal concentrations of salts. Contents of $K^+$, $Na^+$, $Mg^{2+}$ and $Ca^{2+}$ on a chlorophyll basis were determined by atomic absorption spectroscopy (Table I). The sorbitol-impermeable and water-permeable spaces of intact chloroplasts were determined as 20 µl/mg Chl. The internal cation concentrations calculated are presented in the last column of Table I. Note that *Bryopsis* chloroplasts contain an extremely high concentration of $K^+$. Concentrations of $Na^+$ and $Mg^{2+}$ are also higher than those reported with land plants or fresh-water algae [12,13,16,18]. The $Mg^{2+}$ content is 3-times higher than that of $Ca^{2+}$.

The thylakoid membranes and proteins inside the intact chloroplasts carry net negative charges on their surface and thus attract cations into the diffuse layer immediately adjacent to the surface [19]. Divalent cations such as $Mg^{2+}$ and $Ca^{2+}$ may form stable complexes with various organic and inorganic compounds. Thus, the effective concentrations of cations inside chloroplasts should be lower than their total concentrations estimated above. Because the internal level of cations is very important in understanding the regulatory effects of cations on various photosynthetic processes of the thylakoid and stroma, we have attempted to determine the states of cations in *Bryopsis* chloroplasts.

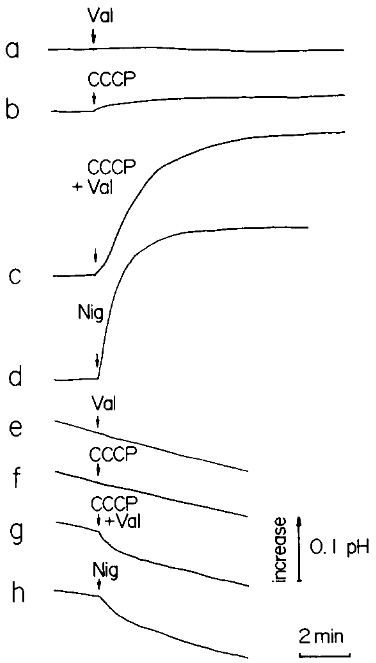

Fig. 2. pH changes induced by the addition of ionophores and CCCP to chloroplast suspensions. Chloroplasts were suspended in a medium (1.8 ml) containing 0.95 M sorbitol, 100 mM KCl, 0.5 mM Hepes and 0.5 mM Mes. The time courses of pH changes were determined at 10°C in the dark with continuous stirring. The medium pH values were 6.5 (a–d) and 8.0 (e–h). Where indicated by arrows, 5.5 µM valinomycin (Val), 5.5 µM CCCP or 2.8 µM nigericin (Nig) was added.

TABLE I

CATION CONTENTS OF INTACT *BRYOPSIS* CHLOROPLASTS

Cation contents of ashed chloroplasts were determined with a Shimazu AA-640-12 atomic absorption spectrophotometer. Corrections were made for the intactness of chloroplasts. Mg content was also corrected for that of chlorophyll. Results are expressed as the mean ± S.E. Figures in parentheses indicate numbers of determinations repeated with different chloroplast preparations.

| | Content (µmol/mg Chl) | Concentration (mM) |
|---|---|---|
| $K^+$ | 9.6 ± 0.7 (5) | 470 ± 30 |
| $Na^+$ | 1.04 ± 0.09 (4) | 51 ± 4 |
| $Mg^{2+}$ | 0.76 ± 0.05 (5) | 37 ± 2 |
| $Ca^{2+}$ | 0.26 ± 0.04 (3) | 13 ± 2 |

*Permeability of the outer limiting membrane to ions.* The method described in the Introduction assumes that ionophore-induced transmembrane movement of $H^+$ is tightly coupled with countertransport of a cation. This can be realized only when the outer limiting membrane of chloroplasts is impermeable to ions. Experiments carried out to test the permeability of the membrane are shown in Figs.

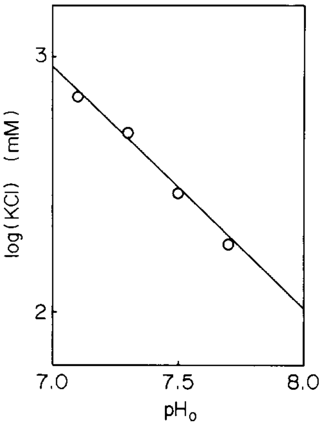

Fig. 3. Relationship between $pH_0$ and KCl concentration where no pH change occurs on addition of nigericin. At the indicated $pH_0$, the pH changes induced by the addition of 2.8 $\mu$M nigericin were determined in the presence of various concentrations of KCl and the KCl concentration giving no pH change was estimated by graphical interpolation.

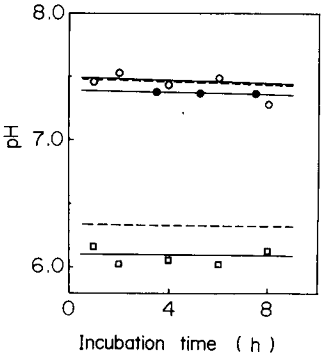

Fig. 4. The internal pH values estimated by the isotope and the Triton methods. Internal pH values were determined after the indicated periods of dark incubation of chloroplasts at 0°C. pH of the stroma (○) and intrathylakoid (□) spaces, determined with the isotope method. (- - - - - -) pH recalculated on the assumption that 30% of protonated methylamine is in the bound state (see Discussion). (●) Internal pH estimated by the Triton method.

2–4. Chloroplasts were suspended in a medium containing low concentrations of buffers and 100 mM KCl. There was no significant pH change on addition of an $H^+$ carrier, CCCP, to the chloroplast suspensions both at pH 6.5 and 8.0 (Fig. 2). This suggests that the outer limiting membrane is impermeable to ions so that, in spite of the pH difference between the intrachloroplast space and the outer medium, massive transport of $H^+$ is prevented by the electrical potential difference formed across the membrane. In fact, when the membrane was made permeable to both $H^+$ and $K^+$ on the combined addition of CCCP and valinomycin, a large pH increase occurred at pH 6.5 and a small but significant decrease at pH 8.0. Similar pH changes were observed on addition of nigericin which facilitates transmembrane exchange of $H^+$ and $K^+$. The results clearly indicate that the outer limiting membrane is impermeable to ions and, therefore, $H^+$ transport across the membrane occurs only when exchange of $H^+$ with $K^+$ is possible.

Thus, at a given $pH_0$, the extents and sign of the pH changes induced by the addition of CCCP plus valinomycin or nigericin depend upon the external concentrations of KCl. No pH change occurs at a certain KCl concentration where the electrochemical potential difference of $H^+$ across the outer limiting membrane is balanced by that of $K^+$. Such KCl concentrations are presented as a function of $pH_0$ in Fig. 3. The slope of unity indicates that a linear relationship between $\log([H^+]_i/[H^+]_o)$ and $\log([K^+]_i/[K^+]_o)$, as predicted from Eqn. 4, does indeed hold.

*Internal pH.* The $pH_i$ (stroma pH) determined with radioactive methylamine and DMO varied in the range 7.2–7.7 with different chloroplast preparations. The pH inside the thylakoid membrane was always lower by more than 1 pH unit than that of the stroma space (Fig. 4). This agrees with a recent observation of Enser and Heber [26] with dark-adapted intact spinach chloroplasts. pH values of the intrachloroplast compartments were fairly constant when chloroplasts were kept at 0°C in the dark in this chloroplast preparation.

*Internal concentrations of $K^+$ and divalent cations.* The pH of the chloroplast suspension was adjusted to that of the stroma and pH changes induced by the addition of nigericin were determined in the presence of varied concentrations of KCl. Fig. 5A shows that no pH change occurred in the presence of 240 mM KCl, indicating that the effective concentration of $K^+$ inside chloroplasts is equivalent to 240 mM. Determinations with three different chloroplast preparations gave a mean value of 260 ± 40 mM (Table II). Fig. 5B shows the $Mg^{2+}$ concentration dependence of the pH changes induced by A23187 which facilitates exchange of $H^+$ and a divalent cation across the mem-

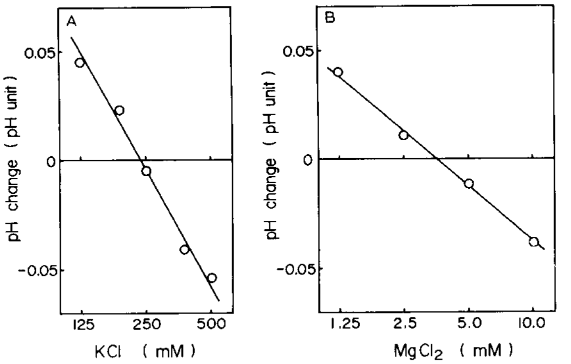

Fig. 5. Extents of pH changes induced by the addition of nigericin in the presence of various concentrations of KCl (A) and by the addition of A23187 in the presence of various concentrations of $MgCl_2$ (B). The reaction media contained the indicated concentrations of KCl or $MgCl_2$. The medium pH was adjusted to be equal to the stroma pH (pH 7.56) determined with the same chloroplast preparation. A, 5.5 μM nigericin. B, 11 μM A23187. Note that the abscissae indicate the logarithm of the salt concentrations.

brane. Note that the slope of the line is one-half of that in Fig. 5A. This reflects the situation that A23187 facilitates $2H^+$-$Mg^{2+}$ exchange across the membrane. The zero intercept of the straight line occurred at 3.6 mM $MgCl_2$, suggesting that only a small fraction of divalent cations is dissolved in the bulk aqueous phase of the chloroplast interior (see Table II).

### *A modified method for determination of the internal concentration of cations*

*pH changes caused by disruption of chloroplast structure.* Determination of $pH_i$ by the procedure above is based on the untested assumption that the intrathylakoid space occupies 12.5% of the total inner space of *Bryopsis* chloroplasts. Since it is essential for this method to adjust $pH_o$ to be equal to $pH_i$, we attempted to investigate $pH_i$ by another method of a different principle.

Addition of a nonionic detergent, Triton X-100, to a chloroplast suspension containing low concentrations of buffers caused pH changes, the sign and magnitude of which varied markedly depending upon the medium pH. When the extents of the pH changes determined were plotted against $pH_o$, a straight line which intercepted the zero-pH-change line at $pH_o$ 7.47 was obtained (Fig. 6). Whether the pH thus titrated represents $pH_i$ was determined in the following.

Triton-induced pH changes at $pH_o$ 6.7 reached the maximum extent at about 0.05% Triton and remained constant at higher concentrations (Fig. 7). Note that pH changes were accompanied by approximately parallel increases in buffering capacity of the suspensions. This indicates that the outer limiting membranes of chloroplasts were disrupted in the presence of 0.05% Triton so that the stroma and the thylakoid membrane contribute to the total buffering capacity.

TABLE II

EFFECTIVE CONCENTRATIONS AND THERMODYNAMIC ACTIVITIES OF CATIONS IN INTACT *BRYOPSIS* CHLOROPLASTS

Results are expressed as the mean ± S.E. Figures in parentheses indicate numbers of determinations repeated with different chloroplast preparations.

| Cation | $pH_i$ determined by | | | | | |
|---|---|---|---|---|---|---|
| | Isotope method | | | Triton method | | |
| | Cation concentration (mM) | % of total concentration | Activity (mM) | Cation concentration (mM) | % of total concentration | Activity (mM) |
| $K^+$ | 260 ± 40 (3) | 55 | 180 | 320 ± 20 (5) | 70 | 220 |
| Divalent cations | 2.7 (2) | 5.4 | 1.9 | 3.1 ± 0.2 (4) | 6.2 | 2.1 |
| $Mg^{2+}$ | | | | 2.5 (2) | 6.8 | 1.7 |
| $Ca^{2+}$ | | | | 0.6 (2) | 4.6 | 0.4 |

Further disintegration of chloroplast structure, as indicated by progressive decreases in light scattering of chloroplasts with increasing concentrations of the detergent up to 0.2%, had little effect on the extent of pH changes and the buffering capacity of the chloroplast suspension. The results indicate that the pH changes observed are due to removal of the barrier separating the medium and the intrachloroplast space.

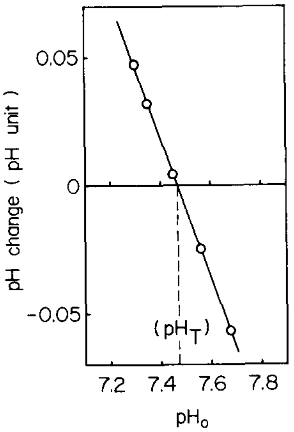

Fig. 6. Dependence on the external pH of the magnitudes of the Triton X-100-induced pH changes. Chloroplasts were suspended in a medium containing 50 mM KCl, 1.0 M sorbitol, 0.5 mM Hepes and 0.5 mM Mes. The medium pH was adjusted to the indicated values with NaOH or HCl. The final concentration of Triton X-100 added was 0.055%.

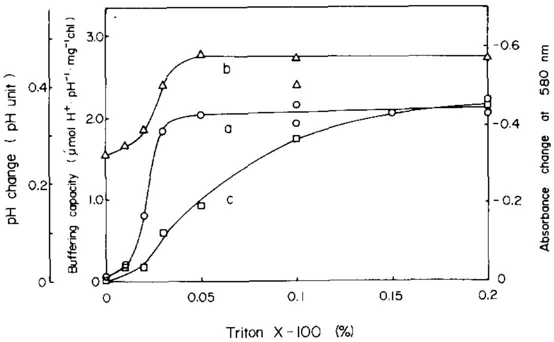

Fig. 7. Effects of Triton X-100 on pH (a), buffering capacity (b) and light scattering (c) of chloroplast suspensions. Chloroplasts were suspended in a medium containing 0.9 M sorbitol, 50 mM KCl and 0.5 mM Hepes. The initial pH of the suspension was 6.7. Light-scattering changes were determined as the apparent absorbance (turbidity) changes at 580 nm with a Hitachi 356 spectrophotometer in the split-beam mode. Magnitudes of changes in pH, buffering capacity and light scattering were determined 5 min after the addition of Triton X-100.

*Effects of cations on Triton-induced pH changes.* However, the pH titrated with the detergent as illustrated in Fig. 6 was found to vary depending upon the concentrations and valence of cations present in the medium. For simplicity, we refer to the pH titrated with Triton as $pH_T$ to distinguish it from the true $pH_i$. Fig. 8A shows that $pH_T$ was lowered with increasing concentrations of KCl and reached a constant level at about 200 mM. NaCl appears to be somewhat more effective than KCl for unknown reasons. A more pronounced decrease in $pH_T$ occur-

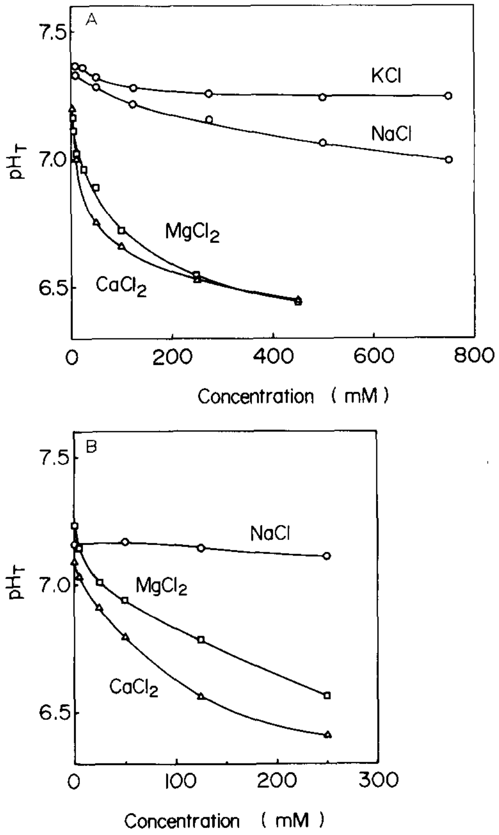

Fig. 8. Effects of salts on the extents of pH changes induced by the addition of Triton X-100. $pH_T$ was determined as in Fig. 6. (A) Chloroplasts were suspended in media containing 0.5 mM Hepes, 0.5 mM Mes and the indicated amount of salts. (B) Same as A plus 250 mM KCl. Appropriate amounts of sorbitol were added to make the osmolality of media 1.3 osmol/kg water. The final concentration of Triton X-100 added was 0.055%.

red in the presence of $MgCl_2$ and $CaCl_2$. Even in the presence of a saturating concentration of KCl, addition of $MgCl_2$ and $CaCl_2$ caused considerable decreases in $pH_T$ (Fig. 8B). Since all the salts added were chloride salts, we conclude that the observed effects of the salts are due to cations and that divalent cations are more effective than monovalent cations.

Bose and Hoch [27] and Masamoto et al. [28] reported that addition of cations to isolated thylakoids caused acidification of the suspensions. The above observations suggest, therefore, that besides $H^+$ in the bulk aqueous phase of the intrachloroplast space, $H^+$ bound to the surface of the thylakoid membranes and proteins present inside chloroplasts contributes to Triton-induced pH changes. When the membranes and proteins are exposed to the external medium containing high concentrations of cations, $H^+$ will be liberated in exchange for the cations [27, 28]. Divalent cations are known to be much more effective in shielding the negative surface charges of thylakoid membranes [3,19,20,28].

*The lower limit for the internal $K^+$ activity.* In order to cancel out the contribution of 'bound' $H^+$, disruption of the outer limiting membrane has to be carried out in suspending medium having a cation composition identical to that of the bulk aqueous phase inside chloroplasts. However, cation concentrations in the intrachloroplast space are not known. Actually, it is the aim of the present work to determine the internal cation levels. We have therefore made the following stepwise approach to this end.

In Fig. 6, $pH_T$ was determined in the presence of 50 mM KCl, because the time courses of the pH changes were too sluggish to be determined accurately in the absence of any cation. This concentration of KCl can be safely regarded as being lower than the cation concentrations inside *Bryopsis* chloroplasts. Thus, the pH changes observed are the result of the mixing of the external and internal aqueous phases and of $H^+$ binding to the surface of the membranes and proteins exposed to the suspending medium. The $pH_T$ determined in Fig. 6 should be, therefore, higher than the true $pH_i$.

Subsequently, we adjusted the medium pH to 7.47 and measured pH changes induced by the addition of nigericin in the presence of various concentrations of KCl. The line obtained by plotting the extents of pH changes against the external KCl concentrations intercepted the zero-pH-change line at 230 mM (Fig. 9). At this KCl concentration, $K^+$ must have been extruded from chloroplasts to induce counterinflux of $H^+$ which compensates the $H^+$ efflux due to $pH_i < pH_o$. Therefore, $[K^+]_o < [K^+]_i$.

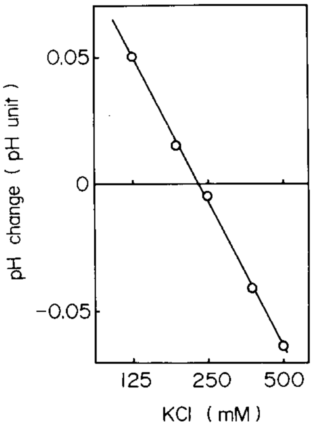

Fig. 9. Plot of magnitudes of nigericin-induced pH changes vs. the external KCl concentrations.

*$pH_i$.* As shown in Fig. 8, 230 mM KCl is saturating with respect to the effect of monovalent cations on Triton-induced pH changes. Thus, the difference between the true $pH_i$ and $pH_T$ determined in the presence of KCl at 230 mM or above arises solely from the difference in divalent cation levels across the outer limiting membrane. It follows that $pH_T$ determined in the presence of 250 mM KCl, with or without 50 mM $MgCl_2$, which corresponds to the total divalent cation concentration inside the chloroplasts (see Table I), set the upper and lower limits for $pH_i$. Then titrations with A23187 at these two $pH_o$ values will in turn give the lower and upper limiting concentrations of divalent cations. Fig. 10 shows that no net pH change occurred on addition of the ionophore in the presence of 2.7 and 4.8 mM $MgCl_2$ at $pH_o$ 7.1 and 6.6, the upper and lower limits for $pH_i$, respectively.

We repeated $pH_T$ measurements in the presence of 250 mM KCl and 2.7 or 4.8 mM $MgCl_2$ to confine $pH_i$ to a narrower range. The two $pH_T$ values thus obtained coincided with each other within the limits of experimental precision to give $pH_i$ 7.0 (data not shown).

$pH_i$ determined by this method again varied with chloroplast preparations between 6.8 and 7.6. Fig. 4

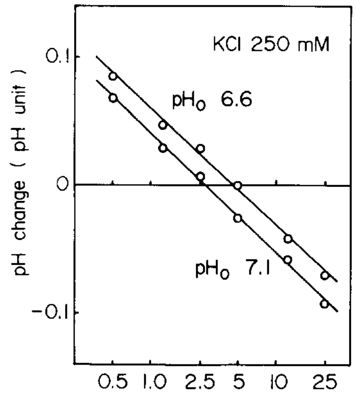

Fig. 10. Plot of magnitudes of A23187-induced pH changes vs. the external $MgCl_2$ concentrations.

shows that $pH_i$ determined by the Triton method is about 0.1 pH unit lower than the stroma pH determined by the first isotope method with the same chloroplast preparation.

*Internal concentrations of free cations.* The internal activities of $K^+$ and divalent cations were estimated using the $pH_i$ determined above. A titration with nigericin showed that the internal concentration of $K^+$ is equivalent to 330 mM KCl in the medium (Fig. 11A). Note that the valinomycin-CCCP couple gave an essentially similar result. The titration with A23187 gave a value of 2.5 mM for the divalent cation concentration with both $MgCl_2$ and $CaCl_2$ (Fig. 11B). The mean value of 320 mM $K^+$ was obtained from five determinations and 3.1 mM $Mg^{2+}$ from four determinations. Assuming the activity coefficients for $K^+$ and $Mg^{2+}$ in 320 mM KCl plus 3.1 mM $MgCl_2$ at 10°C to be 0.68 [29], the thermodynamic activities of $K^+$ and divalent cations inside chloroplasts are estimated to be 220 and 2.1 mM, respectively (Table II).

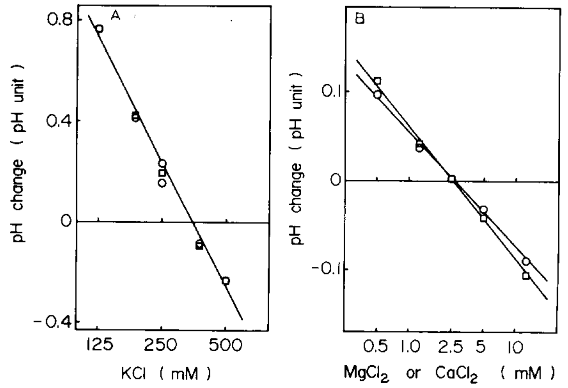

Fig. 11. (A) Plot of magnitudes of pH changes induced by the addition of nigericin, or valinomycin plus CCCP, vs. external KCl concentrations. The medium contained 3.0 mM $MgCl_2$ and was adjusted to pH 7.0. (○) 2.8 $\mu$M nigericin, (□) 5.5 $\mu$M valinomycin plus 5.5 $\mu$M CCCP. (B) Plot of magnitudes of pH changes induced by the addition of A23187 vs. external concentrations of $MgCl_2$ or $CaCl_2$. The medium contained 250 mM KCl. The initial pH was 7.0. 11 $\mu$M A23187 was added. (○) $MgCl_2$, (□) $CaCl_2$.

*Internal concentrations of $Mg^{2+}$ and $Ca^{2+}$.* The time courses of A23187-induced pH changes in the presence of $MgCl_2$ were found to be markedly different from those determined in the presence of $CaCl_2$ (Fig. 12). A23187 caused a faster pH decrease in the presence of 5 mM $CaCl_2$ than in the presence of 5 mM $MgCl_2$. With 2.5 mM $MgCl_2$, there was a transient pH increase, whereas the same concentration of $CaCl_2$ caused a transient pH decrease. The final pH levels attained were similar to each other, however. These kinetic features indicate that the ionophore mediates $Ca^{2+}$-$2H^+$ exchange more rapidly than $Mg^{2+}$-$2H^+$ exchange. Thus, a rapid $H^+$ transport across the outer limiting membrane coupled with uptake or release of $Ca^{2+}$ occurred first, followed by a slower $H^+$ movement in exchange for $Mg^{2+}$. A lag of the pH increase observed in the presence of 1.25 mM $CaCl_2$ suggests that the internal concentration of free $Ca^{2+}$ is lower than that of 1.25 mM $CaCl_2$.

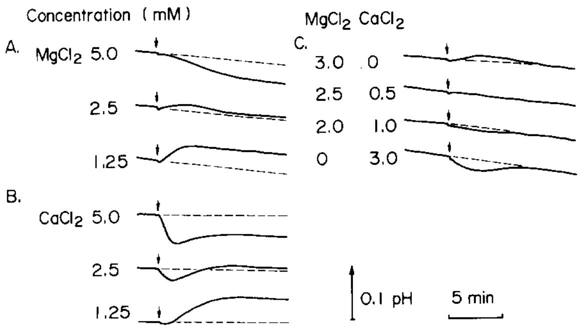

Fig. 12. Time courses of pH changes induced by the addition of A23187 in the presence of different concentrations of $MgCl_2$ or $CaCl_2$. The initial $pH_0$ was 7.0. The medium contained 250 mM KCl. Arrows indicate when 11 $\mu$M A23187 was added.

The above observations prompted us to determine the kinetics of pH changes in the presence of varied combinations of $Mg^{2+}$ and $Ca^{2+}$ concentrations, while keeping the total divalent cation concentration constant at 3 mM. Fig. 12C shows that transient changes of the time course virtually disappeared at a concentration ratio of $Mg^{2+}$ to $Ca^{2+}$ of 5. This indicates that the internal concentrations of $Mg^{2+}$ and $Ca^{2+}$ are 2.5 and 0.5 mM, respectively.

## Discussion

The internal concentrations of cations in *Bryopsis* chloroplasts determined in the present work are significantly higher than those reported with other plants. In terms of the total concentration, the $K^+$ content of the algal chloroplasts far exceeds those determined with land plants and fresh-water algae which range from 20 to 110 mM [12,13,16,18], with the exception of the high values reported by Larkum [15] for four plants. This seems to be compatible with the observation that *Bryopsis* chloroplasts require a higher sorbitol concentration to exhibit maximum photosynthetic activity than intact chloroplasts isolated from other plants. The maximum rate of $O_2$ evolution was attained at a high medium osmolality, which is comparable to that of seawater (1.0 osmol/kg water). This suggests that a high internal concentration of salts is important in maintaining the functional integrity of the organelles of *Bryopsis* which lives in the high salinity of seawater.

However, the internal cation concentrations are still insufficient to give rise to a high internal osmolality comparable to that of seawater (see below). Presumably, *Bryopsis* chloroplasts additionally contain a large amount of uncharged solutes such as sugars.

The present work reports the thermodynamic activity of cations inside intact chloroplasts for the first time. Two sets of results were obtained depending upon the procedures employed for $pH_i$ determination. The first isotope method is based on the assumption that the intrathylakoid space occupies 12.5% of the total intrachloroplast space. This value was obtained by Heldt et al. [21] from the planimetric estimation of electron micrographs of intact spinach chloroplasts. This method may not be very exact and, furthermore, the intrathylakoid space of *Bryopsis* chloroplasts may not be the same as that of spinach chloroplasts. In addition, the distribution of the free methylamine cation inside intact chloroplasts may not be so simple, as was assumed in the original method [21] (see below).

We have developed the second technique which estimates $pH_i$ by measuring the pH changes resulting from Triton-induced disruption of the outer limiting membrane of chloroplasts in a cation environment equivalent to that in intact chloroplasts. The Triton treatment of chloroplasts caused a notable increase in the total buffering capacity of the suspension (Fig. 7). However, this does not affect our results, since $pH_i$ is estimated by interpolating $pH_o$ where no net pH change occurs.

The $pH_i$ determined by both techniques varied considerably with chloroplast preparations. The reason for this variation is not clear. It is our experience, however, that the variation was more marked among different batches of the alga collected at different times of year than within a single batch of the sample. In addition, $pH_i$ determined by the second technique was always lower than the stroma pH determined by the first (see Fig. 4). This may be attributed partly to the situation that the second technique measures only an average pH of the chloroplast interior, as the detergent affects the permeability of the thylakoid membrane as well. Eqn. 4 indicates that an overestimation of $pH_i$ by 0.1 pH unit results in 20% underestimation of the cation activity. Thus, the isotope technique gave lower thermodynamic activities of cations than did the Triton technique (Table II). Because of the uncertainty of the isotope technique discussed above, and of a possible effect of the ionophore (and CCCP) to redistribute $H^+$ across the thylakoid membrane and hence to lower the stroma pH, we consider that the set of values obtained with the Triton method gives a better estimate.

The method employed for determination of the internal activities of cations premises that the outer limiting membrane of chloroplast is impermeable to ions. The results presented in Figs. 2–5 indicate that this is the case. Additional support for this is provided by the observations that nigericin, which mediates an electroneutral exchange of $H^+$ and $K^+$, and the valinomycin-CCCP couple, which transports $H^+$ and $K^+$ according to $\Delta\psi$ as well as $\Delta[H^+]$ and $\Delta[K^+]$, gave the same $K^+$ activities (Fig. 11A) and that the membrane was impermeable to $Cl^-$ as tested by the osmotic method of Gimmler et al. [18] (data not presented).

In this connection, it should be mentioned that the outer limiting membrane of *Bryopsis* chloroplasts is not the envelope in the usual sense. A preliminary study with an electron microscope revealed that, like chloroplasts isolated from other siphonaceous algae [30], each chloroplast has an additional outer membrane which closely coats the outside of the envelope. This coating membrane would provide an additional barrier to ions. It was found also that chloroplasts contain cytoplasmic contamination in narrow gaps between the outer coat and the envelope. However, the amounts of the contaminants were generally so small that they should not interfere significantly with estimation of the internal pH or cation activity.

The most important results obtained in the present work are that large portions of cations present in intact chloroplasts are in some ways in 'bound' states. The effective concentration of $K^+$ corresponds to about 70% of the total $K^+$ concentration. This indicates that a large amount of $K^+$ is attracted into the diffuse layer adjacent to the surface of the thylakoid membranes and proteins. A significant part of $Na^+$ may also be bound to the fixed negative surface charges.

The situation is more striking with divalent cations. The effective concentrations of divalent cations amount to only 5–7% of the total divalent cation concentrations (Table II). Obviously, most of the $Mg^{2+}$ and $Ca^{2+}$ is either trapped in the electric diffuse layer or in association with various organic and inorganic compounds present inside chloroplasts.

$H^+$ is also concentrated in the electric diffuse layer at the surface of the thylakoid membranes and proteins and is liberated on addition of cations (Fig. 8). The maximum amount of $H^+$ liberated was estimated as 0.6 μequiv./mg Chl which is comparable to 0.4–0.6 μequiv./mg Chl reported with dwarf pea chloroplasts [27] and 0.5 μequiv./mg Chl with spinach chloroplasts [28]. However, *Bryopsis* chloroplasts require much higher concentrations of divalent cations to cause maximum release of $H^+$ than do higher plant chloroplasts. Presumably, *Bryopsis* thylakoid membranes have a higher density of negative surface charges than spinach or pea thylakoids.

The estimation of internal pH by the isotope method of Heldt et al. [21] assumes that methylamine equilibrates across the outer limiting membranes depending upon the pH of the medium and of the chloroplast compartments. The present work indicates, however, that the negative surface charges of the thylakoid membranes and proteins also affect the distribution of the protonated amine between the outer medium and the chloroplasts. Assuming that 30% of the amine cation present inside chloroplasts is attracted into the double layer adjacent to the surface of the membranes and proteins, we reestimated the internal pH values and the results are presented in Fig. 4. It can be seen that the pH gap between the stroma and the intrathylakoid spaces is significantly narrowed by an increase in the intrathylakoid pH. On the other hand, the stroma pH was only slightly affected.

The methods developed in the present work would be useful to determine the thermodynamic activities of monovalent and divalent cations as well as the pH inside intact chloroplasts from other plants, or in other cellular organelles. Preliminary experiments indicated that the methods can be applied, with some minor modifications, to intact chloroplasts from spinach with an envelope membrane more permeable to ions than that of *Bryopsis* chloroplasts.

## Acknowledgements

The authors wish to thank Dr. S. Murakami for the electron microscopic inspection of *Bryopsis* chloroplasts, and Dr. K. Watanuki and Dr. B. Takano for their advice during the atomic absorption spectrophotometry. Ionophores A23187 and nigericin were kind gifts of Dr. R.L. Hamill, Eli Lilly and Company, Indianapolis and Dr. M. Nishimura, Kyushu University, respectively. The present work was partly supported by the Toray Science and Technology Grant and by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, to K.S. (No. 454221) and S.K. (Nos. 511614, 510405, 510307).

## References

1 Murata, N. (1969) Biochim. Biophys. Acta 189, 171–181
2 Gross, E.L. and Hess, S.C. (1973) Arch. Biochem. Biophys. 159, 832–836

3 Barber, J., Mills, J. and Love, A. (1977) FEBS Lett. 74, 174–181

4 Krause, G.H. (1974) Biochim. Biophys. Acta 333, 301–313

5 Briantais, J.-M., Vernotte, C., Picaud, M. and Krause, G.H. (1979) Biochim. Biophys. Acta 548, 128–138

6 Shavit, N. and Avron, M. (1967) Biochim. Biophys. Acta 131, 516–525

7 Sun, A.S.K. and Sauer, K. (1972) Biochim. Biophys. Acta 256, 409–427

8 Dilley, R.A. and Vernon, L.P. (1965) Arch. Biochem. Biophys. 111, 365–375

9 Hind, G., Nakatani, H.Y. and Izawa, S. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 1484–1488

10 Baier, D. and Latzko, E. (1975) Biochim. Biophys. Acta 396, 141–148

11 Badger, M.R. and Lorimer, G.H. (1976) Arch. Biochem. Biophys. 175, 723–729

12 Saltman, P., Forte, J.G. and Forte, G.M. (1963) Exp. Cell Res. 29, 504–514

13 Kishimoto, U. and Tazawa, M. (1965) Plant Cell Physiol. 6, 507–514

14 Harvey, M.J. and Brown, A.P. (1969) Biochim. Biophys. Acta 172, 116–125

15 Larkum, A.W.D. (1968) Nature 218, 447–449

16 Nobel, P.S. (1969) Biochim. Biophys. Acta 172, 134–143

17 Lin, D.C. and Nobel, P.S. (1971) Arch. Biochem. Biophys. 145, 622–632

18 Gimmler, H., Schäfer, G. and Heber, U. (1974) in Proceedings of the 3rd International Congress on Photosynthesis (Avron, M., ed.), pp. 1381–1392, Elsevier, Amsterdam

19 Nakatani, H.Y., Barber, J. and Minski, M.J. (1979) Biochim. Biophys. Acta 545, 24–35

20 Nakatani, H.Y., Barber, J. and Forrester, J.A. (1978) Biochim. Biophys. Acta 504, 215–225

21 Heldt, H.W., Werdan, K., Milovancev, M. and Geller, G. (1973) Biochim. Biophys. Acta 314, 224–241

22 Jensen, R.G. and Bassham, J.A. (1966) Proc. Natl. Acad. Sci. U.S.A. 56, 1095–1101

23 Heber, U. and Santarius, K.A. (1970) Z. Naturforsch. 25b, 718–728

24 Heldt, H.W. and Sauer, F. (1971) Biochim. Biophys. Acta 234, 83–91

25 Schönfeld, M., Rahat, M. and Neuman, J. (1973) Plant Physiol. 52, 283–287

26 Enser, U. and Heber, U. (1980) Biochim. Biophys. Acta 592, 557–591

27 Bose, S. and Hoch, G.E. (1978) Z. Naturforsch. 33c, 105–107

28 Masamoto, K., Itoh, S. and Nishimura, M. (1980) Biochim. Biophys. Acta 591, 142–152

29 Harned, H.S. and Cook, M.A. (1937) J. Am. Chem. Soc. 59, 1290–1292

30 Grant, B.R. and Wright, S.W. (1980) Plant Physiol. 66, 130–138