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# CORRELATION BETWEEN CALCIUM ION BINDING TO CHLOROPLAST MEMBRANES AND DIVALENT CATION-INDUCED STRUCTURAL CHANGES AND CHANGES IN CHLOROPHYLL *a* FLUORESCENCE

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## SUMMARY

1. Two distinct sites were observed for $Ca^{2+}$ binding to chloroplast membranes. Site I has a number of sites equal to 0.65 $\mu$mole/mg chlorophyll and has a dissociation constant of $8 \pm 3$ $\mu$M. This site is the same as that previously observed for $Mn^{2+}$ (Gross, E. L. (1972) Arch. Biochem. Biophys. 150, 324–329) and is responsible for reversal of quaternary ammonium salt uncoupling (Gross, E. L. (1971) Arch. Biochem. Biophys. 147, 77–84).

2. Site II has a number of sites equal to $0.5 \pm 0.2$ $\mu$mole/mg chlorophyll and a dissociation constant of $51 \pm 8$ $\mu$M. Other divalent cations such as $Mn^{2+}$ and $Mg^{2+}$ also bind to this site.

3. Divalent cation binding to Site II was correlated with divalent cation-induced structural changes.

4. Monovalent cations such as $K^+$ and tetraethylammonium also bind to this site but do not cause structural changes. Another explanation for monovalent cation-induced structural changes must be found.

5. Divalent cation binding was also correlated with divalent cation-induced changes in chlorophyll *a* fluorescence.

6. The divalent cations were found to bind to previously unoccupied sites on the chloroplast membrane rather than to exchange for other cations already present.

## INTRODUCTION

Murata and co-workers [1–3], and Homann [4] have shown that divalent cations such as $Ca^{2+}$ and $Mn^{2+}$ inhibit spillover of excitation energy from Photosystem II to Photosystem I in green plant photosynthesis. Murata [3], Murakami and Packer [5] and Mohanty et al. [6] have correlated the chlorophyll *a* fluorescence changes used to monitor spillover with divalent cation-induced structural changes such as had previously been observed in chloroplasts [7] and isolated grana [8]. These results leave unanswered the following questions: (A) What is the mechanism

which produces the structural changes? (B) What do the structural changes represent on the molecular level? (C) How do the stuctural changes actually cause the changes in distribution of excitation energy between the two photosystems?

In this paper, we have investigated the first question and have found that divalent cation-induced structural and fluorescence changes can be correlated with divalent cation binding to the membranes to the extent of 0.5 $\mu$mole/mg chlorophyll with a dissociation constant of 51 $\mu$M. Future studies will explore the nature of the binding groups, the molecules to which they are attached and finally the interaction of these molecules with other components of the chloroplast membrane.

## MATERIALS AND METHODS

### *Chloroplast isolation*

Chloroplasts were isolated as previously described [9] in 50 mM Tris · $Cl^-$ (pH 7.5) plus 350 mM sucrose after which they were washed once and resuspended in 100 mM unbuffered sucrose. Chlorophyll concentrations were determined according to the method of Arnon [10].

### *$^{45}Ca^{2+}$ binding*

Chloroplasts (at 10 $\mu$g/ml chlorophyll) were suspended in 30 ml of a medium containing 100 mM sucrose plus sufficient un-neutralized Tris base to titrate to pH 8 (usually about 0.15 mM) plus various concentrations of $CaCl_2$ containing $^{45}CA^{2+}$. The average specific activity was 3 mCi/mole $CaCl_2$ added. The chloroplasts were collected by centrifugation for 15 min at 10 000 $\times g$ after which the pellets were suspended in distilled water, plated, and the radioactivity counted using a Nuclear Chicago planchet counter.

### *Correction for $^{45}Ca^{2+}$ trapped in the pellet*

Measurements of the [$^{14}$C]sorbitol and $^3H_2O$-permeable space in the chloroplast pellets were made according to the method of Rottenberg et al. [11] and it was found that the extra-chloroplast space comprised 95–100% of the total chloroplast pellet volume. Thus, the $^{45}Ca^{2+}$-binding data were corrected for pellet trapping by subtracting from the values obtained the amount of $^{45}Ca^{2+}$ contained in a volume of medium equal to that of the pellet. The correction calculated by this method was never greater than 10% of the total counts obtained and was negligible for $CaCl_2$ concentrations less than 200 $\mu$M.

The true external concentrations of $CaCl_2$ were found by subtracting the amount bound to the chloroplasts at equilibrium from the total amount added.

The time dependence of $^{45}Ca^{2+}$ binding was determined using the Millipore filtration technique described previously [12]. Chloroplasts at 10 $\mu$g/ml chlorophyll were suspended in 10 ml of a solution containing 1 mM $CaCl_2$ plus other additions as indicated above for various incubation times after which they were collected on a 0.65-$\mu$m Millipore filter. A correction made for the radioactivity trapped by the filter alone averaged 40% of the total counts observed.

### *Tetra[$^{14}$C]ethylammonium chloride binding*

Tetra[$^{14}$C]ethylammonium cation binding was determined as described above

except that corrections for pellet trapping had to be made for all samples due to the high concentrations of tetraethylammonium chloride employed. The correction varied from 20% of the total counts at low concentrations to 50% at 4 mM. The concentration of tetra[$^{14}$C]ethylammonium bromide added was 10 nCi/ml.

*Determination of pellet cation contents by atomic absorption*

The cation content of chloroplasts was determined according to the procedure described earlier for $Mn^{2+}$ [13] in which chloroplasts (at 10 $\mu$g/ml) were incubated in 30 ml of a solution containing 100 mM sucrose plus sufficient Tris base to titrate to pH 8 plus other salts as indicated after which the chloroplasts were collected by centrifugation at 10 000 $\times g$ for 15 min. The pellets thus obtained were resuspended in 2 ml deionized water plus 0.6 ml 70% $HClO_4$. The chloroplasts were extracted for 24 h at 20 °C in $HClO_4$ after which the denatured chloroplasts were removed by centrifugation. The extracts were analyzed for their contents of $Mn^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $Na^+$ and $K^+$ using a Model 303 Perkin–Elmer atomic absorption spectrophotometer.

*Divalent cation-induced structural changes*

Divalent cation-induced structural changes were determined by monitoring either turbidity changes at 540 nm or by measuring pellet weights as previously described [13]. The structural changes were determined as a function of cation concentration under conditions identical to those used for the cation-binding studies described above.

*Divalent cation-induced changes in chlorophyll a fluorescence*

The effects of divalent cations on chlorophyll *a* fluorescence were determined as previously described [14] using an Aminco–Bowman spectrofluorometer. The excitation and emission wavelengths were 470 and 680 nm, respectively. The reaction mixtures were identical to those used for the binding studies with the following exceptions: (A) The chlorophyll concentration was 5 $\mu$g/ml. (B) The reaction mixture contained 6.7 $\mu$M 3(3,4-dichlorophenyl)-1,1-dimethylurea to insure that the Photosystem II traps were closed. (C) 10 mM NaCl was added to each reaction mixture. The addition of NaCl was necessary in order to promote spillover so that it could be inhibited by further additions of salts of divalent cations (see ref. 15).

*Chemicals*

Trizma base, pyocyanin perchlorate and tetraethylammonium chloride were obtained from Sigma, Schwartz-Mann and the Aldrich Chemical companies, respectively. $^{45}CaCl_2$ and tetra[$^{14}$C]ethylammonium bromide were obtained from New England Nuclear. Other chemicals were of reagent grade.

## RESULTS AND DISCUSSION

Previous studies in this laboratory [12] revealed a binding site on the chloroplast membrane for $Mn^{2+}$ and other cations with a dissociation constant ($K_d$) of $8 \pm 3$ $\mu$M. The number of sites ($n$) was found to be 0.64 $\mu$mole/mg chlorophyll (see ref. 12 and Table I). The binding of divalent cations to this site was correlated with divalent cation reversal of quaternary ammonium salt uncoupling [9]. In this report,

TABLE I

BINDING OF DIVALENT CATIONS TO CHLOROPLASTS: RELATIONSHIP TO STRUCTURAL CHANGES

The values of $n_1$ (the numbers of binding sites for Site I) and $n_1+n_2$ (the total number of binding sites for both sites) as well as $K_1$ and $K_2$ (the dissociation constants for divalent cation binding at Site I and Site II, respectively) were determined from data similar to those presented in Fig. 1. $n_2$ (the number of binding sites for Site II) represents the difference between the total number of binding sites and the number determined for Site I. Each value represents between 5 and 10 separate determinations. $K_i$ (the inhibitor constant for competitive displacement of $Ca^{2+}$ by the cation in question) was determined by Dixon plots as shown in Fig. 2. Each value represents the average of five separate determinations. Turbidity changes ($\Delta A_{546\ nm}$ and pellet weights were determined as a function of $CaCl_2$ concentration after which the concentrations required to observe half-maximal effects ($c_{1/2\ max}$) were calculated. Each value represents an average of five separate determinations. Other conditions were as described in Materials and Methods. The maximum increase in turbidity observed upon addition of high concentrations of $CaCl_2$ averaged 61 %. The average pellet weight determined in the absence of $CaCl_2$ was 130 mg/mg chlorophyll and the average maximal percent decrease was 42 %.

| Cation: | $Ca^{2+}$ | $Mn^{2+}$ | $Mg^{2+}$ | $TEA^{+}$* | $K^+$ |
|---|---|---|---|---|---|
| Cation binding at site I | | | | | |
| $n$($\mu$mole/mg chlorophyll) | 0.65 ±0.11 | 0.64 ±0.15 | | | |
| $k_{d_1}$ (mM) | 0.008±0.003 | 0.009±0.003 | | | |
| Cation binding at site II | | | | | |
| $n_1+n_2$ | 1.2 ±0.3 | 1.2 ±0.1 | | 1.2±0.3 | |
| $n_2$ | 0.5 ±0.2 | 0.6 ±0.2 | | | |
| $K_{d_2}$ (mM) | 0.051±0.008 | 0.054±0.008 | | 2.0±0.5 | |
| $K_{i_2}$ (mM) | | | 0.061±0.012 | 2.6±0.5 | 3.5±0.5 |
| Structural changes $c_{\frac{1}{2}\ max}$ (mM) | | | | | |
| $\Delta A_{540\ nm}$ | 0.060±0.006 | | 0.085±0.015 | 35±7 | 34±5 |
| Pellet weights | 0.070±0.020 | | | | |

* Tetraethylammonium.

we have expanded our studies to include higher divalent cations concentrations in the range required to produce changes in chloroplast structure [13] and chlorophyll *a* fluorescence yields [1–3, 14]. The results are shown in Fig. 1a. It can be seen that a double reciprocal plot of the inverse of $^{45}Ca^{2+}$ binding vs the inverse of the $Ca^{2+}$ concentration gives a plot with two straight line segments indicating two independent cation binding sites. The first site (which occurs at lower $Ca^{2+}$ concentrations and thus has the higher binding affinity) has a dissociation constant of 8±3 $\mu$M and the number of sites is 0.65 $\mu$mole/mg chlorophyll (see also Table I). These values agree very closely with those previously obtained for $Mn^{2+}$ (see ref. 12 and Table I). Therefore, we can conclude that this site is the same as that described previously for $Mn^{2+}$ and is also responsible for reversal of quaternary ammonium salt uncoupling.

The second site which is shown more clearly in Fig. 1b has a dissociation constant of 51 ± 8 $\mu$M and intersects the ordinate at 1.2 $\mu$mole/mg chlorophyll. The true number of binding sites for Site II can be obtained by subtracting the number of binding sites for Site I from this number yielding a value of 0.5±0.2 $\mu$mole/mg chlorophyll (see Table I). The results obtained for $Mn^{2+}$ also shown in Table I

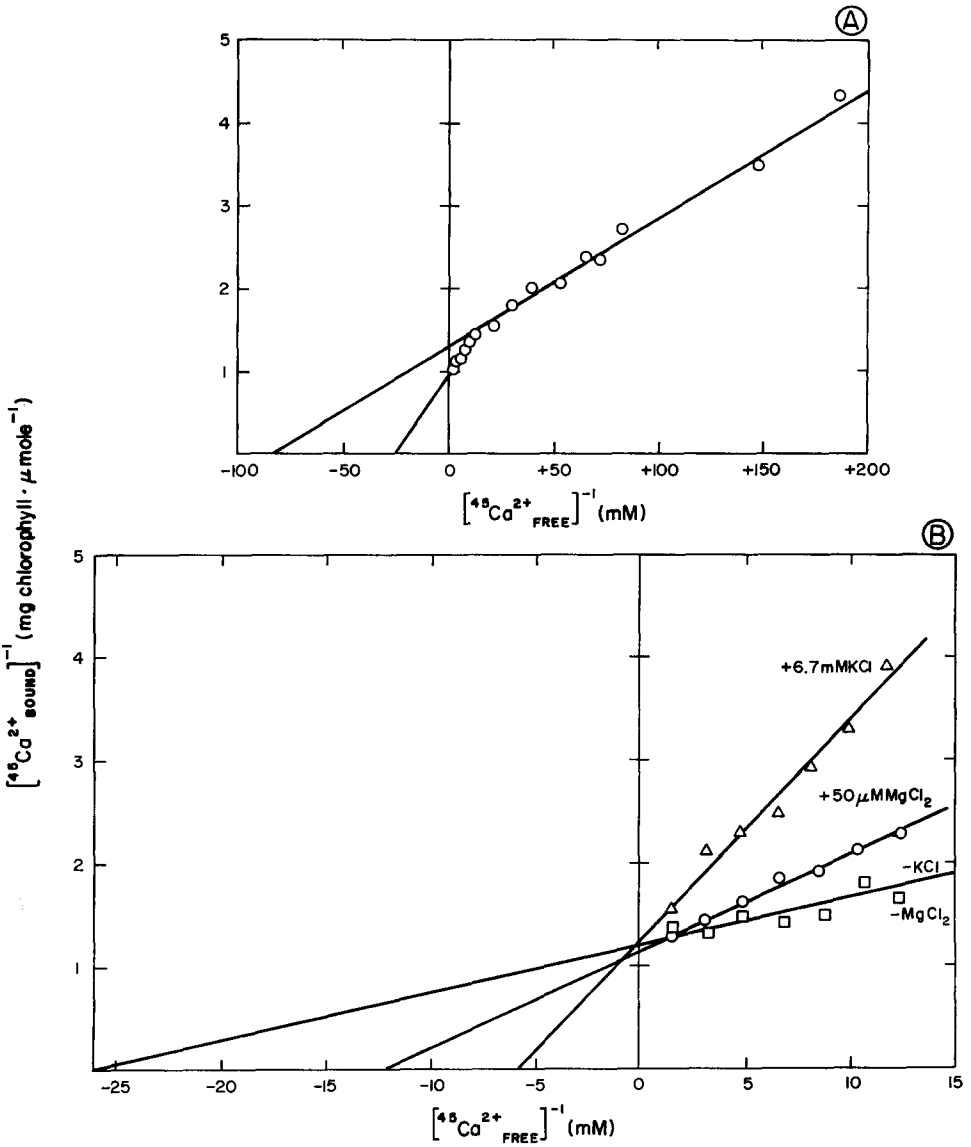


Fig. 1. Concentration dependence of $Ca^{2+}$ binding to chloroplast membranes. (A) The existence of two sites for $Ca^{2+}$ binding. $Ca^{2+}$ binding was determined as a function of $Ca^{2+}$ concentration in the medium. Other conditions were as described in Materials and Methods. A double reciprocal plot was constructed according to the following formula:

$$\frac{1}{[Ca^{2a+}{}_{bound}]} = \frac{1}{n}\left(1 + \frac{K_d}{[Ca^{2+}{}_{free}]}\right)$$

where $n$ is the total number of binding sites; $K_d$ is the dissociation constant and $[Ca^{2+}{}_{free}]$ refers to the $Ca^{2+}$ concentration in the medium at equilibrium. (B) Expanded plot for Site II showing competitive displacement of $Ca^{2+}$ by $Mg^{2+}$ and $K^+$. The conditions were as described in (A) except that either 50 $\mu$M $MgCl_2$ or 6.7 mM KCl were added where as indicated.

indicate that both cations bind to this site with the same binding affinity to within the limit of error.

Fig. 1b also shows that other cations such as $K^+$ and $Mg^{2+}$ can displace $Ca^{2+}$ from the chloroplast membranes. Furthermore, the displacement is competitive in nature since the apparent dissociation constant for $Ca^{2+}$ was increased but the number of sites was unaffected. The competitive nature of the displacement suggests that $Mg^{2+}$ and $K^+$ bind to the same sites as do $Ca^{2+}$ and $Mn^{2+}$. Furthermore, the dissociation constants for the competing cations should be equal to the inhibitor constants ($K_i$) for their displacement of $Ca^{2+}$ from the membranes. The inhibitor constants can be obtained from plots such as the one shown in Fig. 2 for tetraethylammonium chloride. Data was obtained in this manner for KCl, tetraethylammonium chloride and $MgCl_2$ and is summarized in Table I. The results obtained for $Mg^{2+}$ are sufficiently close to those for $Ca^{2+}$ and $Mn^{2+}$ to indicate that there is little or no selectivity for one divalent cation over another. In contrast, monovalent cations have higher inhibitor constants in the range of 2.5–3.5 mM.

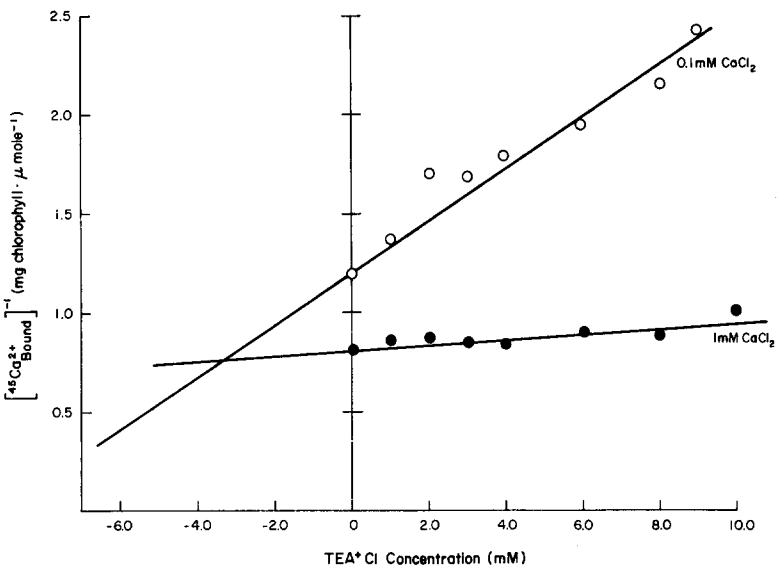


Fig. 2. Displacement of $Ca^{2+}$ from chloroplast membranes by tetraethylammonium cations ($TEA^+$). $Ca^{2+}$ binding was determined as a function of tetraethylammonium chloride concentration in the presence of either 0.1 or 1.0 mM $CaCl_2$. A Dixon plot was made according to the following formula:

$$\frac{1}{[Ca^{2+}_{bound}]} = \frac{1}{n}\left\{1 + \frac{K_d}{[Ca^{2+}_{free}]} \cdot \left(1 + \frac{[TEA^+]}{K_{i\,TEA^+}}\right)\right\}$$

The intersection of the two lines represent the negative of the inhibitor constant ($K_i$) for competitive displacement of $Ca^{2+}$ by tetraethylammonium ions. Other conditions were as described for Fig. 1.

*Direct determination of tetra[$^{14}C$]ethylammonium cation binding*

Direct determination of tetraethylammonium cation binding were made to confirm these results. A typical experiment is shown in Fig. 3 and the results of five

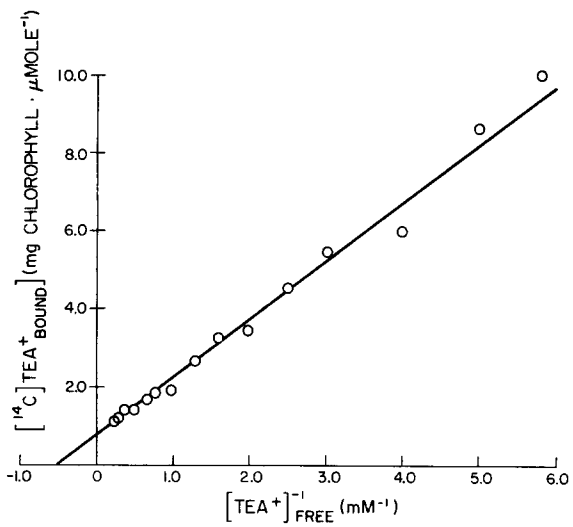


Fig. 3. Binding of tetraethylammonium cations to chloroplast membranes. Tetraethylammonium cation binding to chloroplast membranes was determined as a function of tetra[$^{14}$C]ethylammonium cation concentration after which a double reciprocal plot was made. Other conditions were as for Fig. 1 or as described in Materials and Methods.

experiments are summarized in Table I. One of the most interesting facets of these results is that, unlike the case for divalent cations, no break in the curve was observed suggesting that only one binding site exists for monovalent cations. Moreover, the line extrapolates to $n = 1.2 \pm 0.3$ μmoles/mg chlorophyll which is the same value obtained for divalent cations when both Site I and Site II are added together, or twice the value for Site II alone. There are two possible interpretations of these results. (1) Monovalent cations may not see these two sites as separate sites or put another way, the dissociation constants for monovalent cations for the two sites may be the same to within the limit of error. (2) Monovalent cations may not bind to Site I at all and the total binding capacity of 1.2 μmoles/mg chlorophyll may belong to Site II. This is consistent with the previous observation [12] that monovalent cations displace divalent cations from Site I in a non-competitive manner. If this were true, then it could be that monovalent cations bind to a single negatively charged group on the chloroplast membrane whereas divalent cations by virtue of their double positive charge form a bridge between two negatively charged groups, thus resulting in half the number of binding sites as observed for monovalent cations.

On the other hand, the dissociation constants for tetraethylammonium cation binding obtained by the direct method agree with the inhibitor constants obtained from the Dixon plots confirming that the real value for the dissociation constants for monovalent cations does lie in the 2–4 mM range. The significance of these values will be discussed later when we compare cation binding and structural changes.

*The pH dependence of $Ca^{2+}$ binding*

The pH dependence of $Ca^{2+}$ binding is shown in Fig. 4. It can be seen that lowering the pH value below 8 results both in an increase in the apparent dissociation

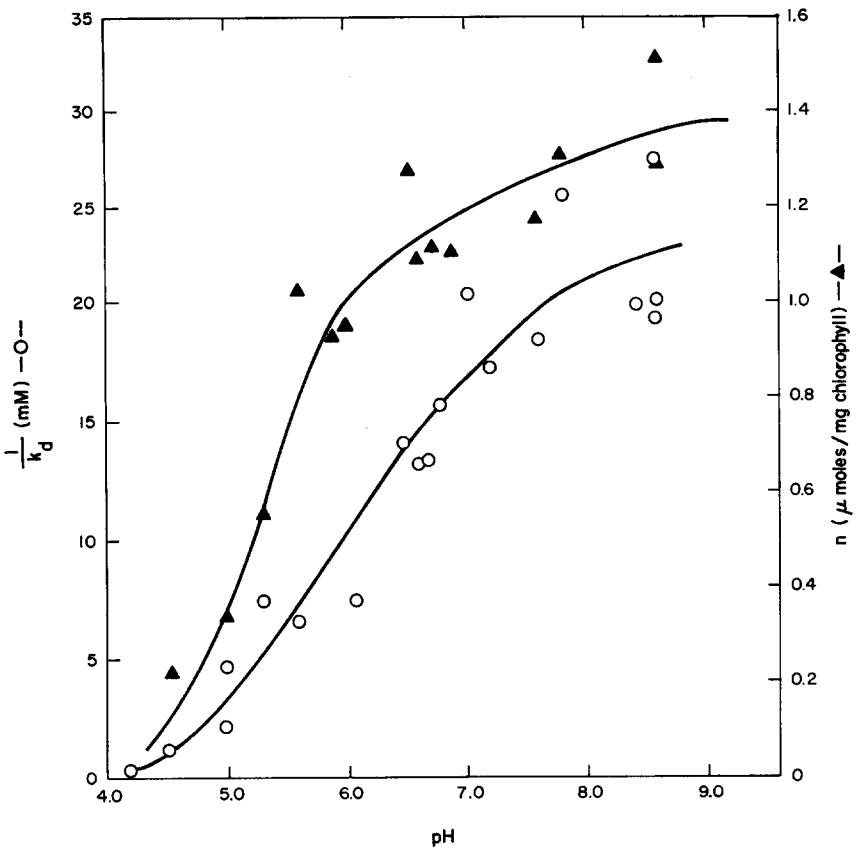


Fig. 4. The effect of pH on $^{45}Ca^{2+}$ binding to chloroplast membranes. A complete concentration curve for $Ca^{2+}$ binding was determined at the indicated pH values after which the number of sites ($n$) and the dissociation constant ($K_d$) were determined from double reciprocal plots similar to those presented in Fig. 1. The pH was adjusted in each case by adding either HCl or unneutralized Tris base. Other conditions were as described for Fig. 1 (except that the 0.15 mM Tris was not included).

constant ($K_d$) and in a decrease in the number of potential binding sites ($n$). The latter effect may be related to structural changes which have previously been observed upon lowering the pH of a chloroplast suspension [13, 15]. It is quite plausible that the structural changes could bury potential binding sites so that they are no longer accessible to $Ca^{2+}$. One possible interpretation of the dissociation constant data is that protons compete for the binding sites. If so, the midpoint of the transition in $K_d$ should be the p$K_a$ for the sites and, in this case, is approximately pH 6.3. However, the transition itself is too broad for there to be a single well-defined p$K_a$ for the sites but rather the data indicate that there are many sites with slightly different p$K_a$ values. This is the same phenomenon that produces the linear pH titration curve for chloroplasts observed in the region of pH 6–8 [16]. It is also possible that the apparent p$K_a$ is due to protonation of other sites which prevent access of $Ca^{2+}$ to the binding sites themselves.

*The effect of light on $Ca^{2+}$ binding*

We have previously shown [13] that illuminating chloroplasts for 1 min under our conditions in the presence of pyocyanin to catalyze cyclic electron flow did not affect the amount of $Ca^{2+}$ bound. These results stand in contrast to those of Nobel and Packer [17] who observed light-induced $Ca^{2+}$ uptake. However, the difference in results may be due to the fact that Nobel and Packer [17] added $Mg^{2+}$ to their reaction mixture and illuminated their samples for 30 min. We can conclude from our studies that the $Ca^{2+}$ binding reported here is not an energy-requiring phenomenon.

*The effect of washing on the ability of chloroplast membranes to bind $Ca^{2+}$*

One important question which arises concerns whether the $Ca^{2+}$ binding observed above is due to the membranes themselves or to soluble proteins and other components which are easily removed from chloroplasts (for example, by washing). If the latter were the case, there could be no correlation between the cation binding, on the one hand, and membrane-dependent phenomena such as structural changes or chlorophyll *a* fluorescence changes on the other. To answer this question, the total $Ca^{2+}$-binding capacity ($n$) was examined under optimal conditions (1 mM $CaCl_2$, pH 8) for sucrose-isolated chloroplasts as a function of the number of washes in 100 mM unbuffered sucrose (Table II). It can be seen that the $Ca^{2+}$-binding capacity of unwashed sucrose-isolated chloroplasts decreased by 50% after the first wash and remained approximately constant thereafter. Equilibrium dialysis measurements (not shown) indicated that the binding entities lost by washing could be recovered from the supernatants and are probably soluble proteins. These results can be explained on the basis that there are two types of binding entities in intact chloroplasts. One type is due to soluble proteins which are lost on the first wash. The second type, which is not lost upon washing in aqueous media (washing in distilled water or 1 mM EDTA produced identical results) represents membrane-bound sites. It is

TABLE II

THE EFFECT OF WASHING CHLOROPLASTS ON THEIR ABILITY TO BIND $Ca^{2+}$

Chloroplasts were isolated in 50 mM Tris (pH 7.5) plus 350 mM sucrose and were washed for the number of times indicated in 100 mM unbuffered sucrose after which their ability to bind $Ca^{2+}$ was determined. The assay medium consisted of 100 mM sucrose plus Tris base (to titrate to pH 8.0) plus 1 mM $CaCl_2$. Other conditions were as described in Materials and Methods. $Ca^{2+}$-induced structural changes were measured by monitoring the absorbance (turbidity) at 540 nm in the presence and absence of 1 mM $CaCl_2$. Other conditions were as described in Materials and Methods. The chloroplasts which were washed once in unbuffered sucrose correspond to those used for the rest of the studies reported in this paper.

| Number of washes | $^{45}Ca^{2+}$ bound ($\mu$moles/mg chlorophyll) | Structural changes $[A_{540\ nm}]+Ca^{2+}$ $[A_{540\ nm}]-Ca^{2+}$ |
|---|---|---|
| 0 | 4.3 | 1.36 |
| 1 | 1.9 | 1.26 |
| 2 | 1.8 | 1.18 |
| 3 | 1.4 | 1.19 |

this latter type of binding that we have examined in this paper since we always use washed chloroplasts.

*The effect of $CaCl_2$ addition on the content of other cations in chloroplasts*

Another question which arises concerns whether the $Ca^{2+}$ binding measured above represents (1) exchange with endogenous $Ca^{2+}$, (2) replacement of other mono- or divalent cations on the binding sites, or (3) binding to previously unoccupied sites. It is this last possibility which is the most interesting when considering the mechanism of the structural changes.

To answer this question, we determined the content of chloroplast $Mg^{2+}$, $Ca^{2+}$, $Na^{2+}$ and $K^+$ in the presence and absence of 0.2 mM $CaCl_2$. The results presented in Table III show that $Ca^{2+}$ addition caused no change in the content of $Mg^{2+}$, $Na^+$ or $K^+$ indicating that exchange with these ions did not occur. Also, the fact that the content of calcium did increase shows that at least part of the calcium added must be binding to previously unoccupied sites. However, some of it could be exchanging with the endogenous $Ca^{2+}$. To test this possibility, we examined the effect of $Mn^{2+}$ and $Na^+$ on the endogenous calcium content of the membranes. Since these ions bind to the same sites as do $Ca^{2+}$, they should displace the endogenous $Ca^{2+}$ if these were bound to the sites of interest. It can be seen (Table IV) that the addition of these ions did not affect the endogenous $Ca^{2+}$ content. Therefore, all of these ions bind to previously unoccupied sites.

It was of interest to compare these results with those obtained by other workers. Nobel and Packer [17] found a slightly lower value for the endogenous $Ca^{2+}$ content, namely 0.25 $\mu$mole/mg chlorophyll and a slightly lower increase in binding (uptake) namely 0.15 $\mu$mole/mg chlorophyll, upon addition of 100 $\mu$M $CaCl_2$. This could be due to the presence of other cations in their reaction medium. Most other workers have determined the cation content of whole chloroplasts isolated either by non-aqueous methods [18] or by rapid centrifugation techniques [19]. Our determinations of the cation contents of unwashed sucrose-isolated chloroplasts pre-

TABLE III

THE EFFECT OF THE ADDITION OF $Ca^{2+}$ TO CHLOROPLAST SUSPENSIONS ON THEIR CONTENT OF OTHER CATIONS

The chloroplasts employed were either unwashed sucrose-isolated chloroplasts or membranes washed once in unbuffered sucrose identical to those used for other studies reported here. They were incubated in a medium containing 100 mM sucrose plus sufficient Tris base to obtain pH 8 in the presence or absence of 0.2 mM $CaCl_2$ after which the pellets were collected by centrifugation and the pellet ion contents determined by atomic absorption. The results represent an average of five separate experiments. The values for $Mg^{2+}$ have been corrected for the $Mg^{2+}$ released from chlorophyll by the extraction procedure.

| Chloroplast preparation | $CaCl_2$ added | Cation content ($\mu$moles/mg chlorophyll) | | | |
|---|---|---|---|---|---|
| | | $Ca^{2+}$ | $Mg^{2+}$ | $Na^+$ | $K^+$ |
| Washed lamellae | None | 0.56±0.07 | 0.8±0.3 | 0.20±0.08 | 0.06±0.01 |
| Washed lamellae | 0.2 mM | 1.10±0.20 | 0.7±0.2 | 0.24±0.08 | 0.05±0.01 |
| Unwashed chloroplasts | None | 1.9 ±0.7 | 2.5±0.4 | 0.45±0.07 | 7.3 ±1.2 |

TABLE IV

THE EFFECT OF THE ADDITION OF $Mn^{2+}$ AND $Na^+$ ON THE CONTENT OF OTHER CATIONS IN CHLOROPLASTS

The pellet cation contents were determined in the presence and absence of the salts indicated. Other conditions were as for Fig. 2.

| Additions | Cation content ($\mu$moles/mg chlorophyll) | | | | |
|---|---|---|---|---|---|
| | $Ca^{2+}$ | $Mg^{2+}$ | $Mn^{2+}$ | $Na^+$ | $K^+$ |
| None | 0.70 | 0.7 | 0.01 | 0.20 | 0.06 |
| 0.2 mM $CaCl_2$ | 1.20 | 0.6 | 0.01 | 0.29 | 0.06 |
| 0.2 mM $MnCl_2$ | 0.67 | 0.5 | 0.69 | 0.23 | 0.07 |
| 10 mM NaCl | 0.57 | 0.5 | 0.01 | 3.0 | 0.07 |

sented in Table IV agree with those obtained by other workers [18, 19] in that (A) $K^+$ is the predominate cation, (2) that the $K^+/Na^+$ ratio is about 10 : 1 and (3) that there are more monovalent than divalent cations present. A comparison of these results with the results obtained for washed membranes shown in Tables III and IV suggests that most of $K^+$ may exist in the stroma fraction of the chloroplasts whereas at least one-third of the $Na^+$, $Mg^{2+}$ and $Ca^{2+}$ may be membrane bound.

*Correlation between divalent cation binding to chloroplast membranes and divalent cation-induced structural changes*

The next question which arises concerns whether the observed divalent cation binding is related to the divalent cation-induced structural changes. If they are related, the following should be true: (1) They should have the same concentration dependence and (2) they should have the same time dependence. Structural changes were determined as a function of cation concentration by either (1) monitoring turbidity changes at 540 nm or (2) determining pellet weights. The conditions used were identical to those used for the binding experiments allowing a direct comparison to be made. The results presented in Table I show good agreement* between binding and structural changes for all three divalent cations tested; namely, $Ca^{2+}$, $Mn^{2+}$ and $Mg^{2+}$. These results indicate that binding of divalent cations to these sites does produce structural changes. However, the correlation does not hold for monovalent cations since the $K_d$ values for binding range between 2 and 4 mM whereas 35 mM concentrations are required to half-saturate the structural changes. This suggests that although monovalent cations can bind to the sites, once bound they cannot produce the structural changes. The unique role of divalent cations in producing structural changes could be due either to their double positive charge or to the possibility of their forming a bridge between two negatively charged groups on the chloroplast

* The concentrations of $Ca^{2+}$ required for half-maximal saturation of the structural changes refers to the total amount of calcium added to the medium whereas the $K_d$ values refer to the concentration of $Ca^{2+}$ remaining in the medium at equilibrium after a certain amount has been bound. Chloroplasts remove approx. 20 % of the $Ca^{2+}$ from the medium under the conditions described. Thus, the concentrations required for half-maximal saturation of the structural changes would be expected to be about 20 % larger than the $K_d$ values.

membrane. Also, we have to find an alternate explanation for monovalent cation-induced structural changes.

The time dependence of $Ca^{2+}$ binding is presented in Fig. 5. As can be seen, the $Ca^{2+}$ binding occurs in two phases, one of which is too rapid to measure and the second of which requires up to 2 min for completion. The first, rapid phases comprises 75% of the total. Similar results including the rapid initial phase followed by the slower increase were previously observed by use for $Ca^{2+}$-induced structural changes [13].

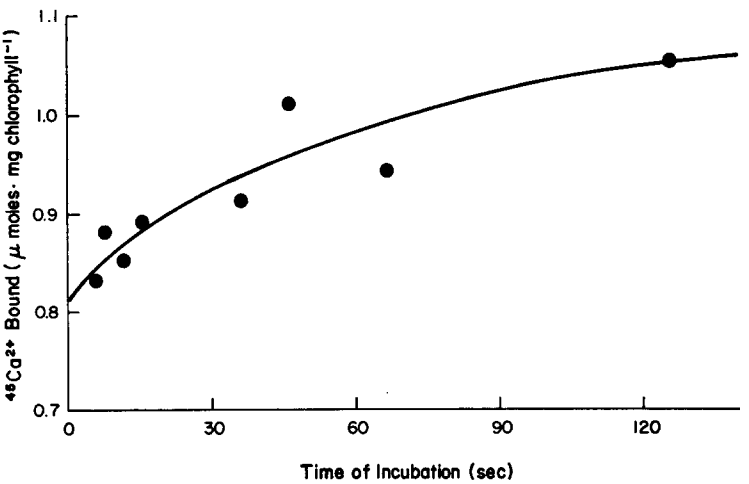


Fig. 5. Time dependence of $Ca^{2+}$ binding. Chloroplasts were incubated for the times indicated in a medium containing 1 mM $CaCl_2$ after which they were collected on a 0.65-$\mu$m Millipore filter. The average time required for filtration was 4 s and the times indicated refer to the point at which 50% of the solution had gone through the filter. Other conditions were as described for Fig. 1.

*Correlation between divalent cation binding and divalent-cation induced changes in chlorophyll a fluorescence*

We have shown above that divalent cation binding can be correlated with the structural changes. Murata [3] and Murakami and Packer [5] have correlated these structural changes with changes in chlorophyll *a* fluorescence indicating a decrease in the spillover of excitation energy from Photosystem II to Photosystem I. In a previous paper [14], we have shown that divalent cation-induced changes in chlorophyll *a* fluorescence do not occur in a medium of low ionic strength such as used for the binding studies described herein since spillover is inhibited under these conditions even in the absence of divalent cations. It was found that 2–10 mM concentrations of salts of monovalent cations promoted spillover, after which further addition of divalent cations reversed the effect inhibiting spillover once more. The observation that structural changes occur in the absence of fluorescence changes does not invalidate the causal relationship between the two. Divalent cations always cause the structural changes but the fluorescence changes only occur when other requirements are also met (namely, the addition of monovalent cations). Therefore, we decided to examine $Ca^{2+}$ binding to membranes under conditions previously used for the

fluorescence studies (namely, in the presence of 10 mM NaCl). Under these conditions the apparent dissociation constant for $Ca^{2+}$ binding was $0.18 \pm 0.05$ mM which agrees with the value of $0.15 \pm 0.03$ mM previously obtained for $Ca^{2+}$-induced fluorescence changes [14]. The increase in the apparent dissociation constant is due to competition for the sites by $Na^{+}$.

## CONCLUSIONS

There are at least two distinct sites for divalent cation binding on the chloroplast membrane. The second site which has an $n$ of 0.6 $\mu$mole/mg chlorophyll and a dissociation constant of $51 \pm 8$ $\mu$M is responsible for divalent cation-induced structural changes. Monovalent cations also bind to these sites but cannot produce structural changes. Under conditions where divalent cation-induced chlorophyll *a* fluorescence changes can be observed (i.e. in the presence of 10 mM NaCl), divalent cation binding can also be correlated with the fluorescence changes. Therefore, divalent cation binding causes both the structural and fluorescence changes when observed under appropriate conditions. However, another explanation must be advanced for the monovalent cation-induced structural changes.

Now that we know that divalent cation binding is the cause of both the structural and fluorescence changes, we can procede to determine the nature of the binding groups and the mechanism by which the structural and fluorescence changes occur.

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