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# THE INFLUENCE OF THE THYLAKOID MEMBRANE SURFACE PROPERTIES ON THE DISTRIBUTION OF IONS IN CHLOROPLASTS

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## Summary

Thylakoid membranes isolated from peas have been subjected to ionic analyses using the technique of neutron activation. This has allowed the analyses of $K^+$, $Na^+$, $Mg^{2+}$, $Ca^{2+}$ and $Cl^-$ to be measured simultaneously on the same sample. By varying the ionic composition of the suspending medium it has been shown that these chloroplast membranes have no obvious chemical specificity for the inorganic cations studied and that the major controlling factor is the electrostatic neutralization of the surface negative charges. In agreement with the Gouy-Chapman theory and for the conditions used, divalent cations were preferentially attracted to the membrane surface. This finding, together with the ionic analysis of the unwashed thylakoids and of isolated intact chloroplasts, indicated that the major physiological surface cation is $Mg^{2+}$ and that $K^+$ is probably the main inorganic cation of the stroma. This conclusion is discussed in terms of counterion movement in response to light induced proton pumping at the thylakoid membrane.

## Introduction

Although there have been several extensive studies to understand the nature of ionic relations of higher plant chloroplasts [1—6] no clear picture has arisen [7]. Nevertheless, ionic regulation of various photosynthetic phenomena has been implicated by a large number of observations, for example; structural changes leading to chloroplast volume changes and grana stacking [8—10], chlorophyll fluorescence yield changes [11—14], enhancement [15], State 1—State 2 changes [16,7], $H^+$-pumping [17—19], electron transport [20—23] and enzyme regulation of the Calvin cycle [24,25].

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Tris, tris-(hydroxymethyl)-aminomethane.

Any attempt to understand how ions distribute themselves in chloroplasts and influence photosynthesis must take into account two important structural features of this organelle. Firstly, the chloroplast has two compartments, the stroma and the intrathylakoid space. The other striking feature is the very large surface area of the thylakoid membranes in relation to the volume of the stromal and intrathylakoid compartments ($10^5$—$10^6$ $cm^{-1}$) [26]. This latter feature becomes particularly significant in regard to how ions distribute themselves in chloroplasts since the thylakoid surface is negatively charged [27—29]. The extent and properties of these surface charges have recently been thoroughly studied using the technique of particle electrophoresis [30]. The existence of fixed negative charges on the thylakoids give rise to a diffuse electrical layer immediately adjacent to the surface [31]. Careful studies have indicated the importance of this diffuse electrical layer in controlling the yield of chlorophyll fluorescence [32—33] and the ability of charged artificial electron acceptors to promote photosynthetic electron flow [34,35]. Analyses of these experiments, together with recent particle electrophoresis measurements [30,31], have emphasised that the ionic composition of the diffuse layer adjacent to the surface is likely to be, under some circumstances, quite different from the bulk solution in which the membranes are suspended. In this paper we report and discuss experiments designed to prove this point and moreover extend our measurements and arguments to gain a firmer understanding of ionic regulation in the intact chloroplast.

## Materials and Methods

Intact chloroplasts were isolated from peas (Feltham first) 8—10 days old by the method previously described [36]. Thylakoid membranes were obtained during this procedure by centrifugation of the supernatant fraction remaining after removal of the intact chloroplasts (5000 × *g* for 5 min). For studies of ion distribution, the isolated thylakoid membranes were treated in the following way. The thylakoids (100 μg chlorophyll/ml) were resuspended in 0.11 M sorbitol brought to pH 7.6 with Tris and having either 20 mM $MgCl_2$ or 100 mM NaCl present. After 15 min incubation, the thylakoids were washed with a 0.11 M sorbitol/Tris solution (pH 7.6) but containing no salt. To study the effect of removing monovalent cations from the surface, the 100 mM NaCl treated thylakoids were equilibrated in 0.11 M sorbitol/Tris, pH 7.6 + 10 mM NaCl to which varying concentrations of divalent cations were added (e.g. $Mg^{2+}$). To study the effect of removal of divalent cations from the thylakoid surfaces, the membranes were treated with 20 mM $MgCl_2$ and then equilibrated in 0.11 M sorbitol/Tris (pH 7.6) having 1 mM $MgCl_2$ present, to which varying concentrations of monovalent ($Na^+$) cations were added. After the various salt additions the thylakoids were incubated in the mixture for 10 min and then centrifuged and washed twice with the salt free 0.11 M sorbitol/Tris (pH 7.6) medium. The averages of triplicate samples were utilized for the figures.

Ionic analysis of intact chloroplasts and thylakoid membranes were carried out using the technique of neutron activation. Samples (200—400 μg chlorophyll) were placed into polythene capsules (1.5 × 0.5 cm) and dried at 60°C. They were then capped and placed into larger polythene capsules (1.8 × 7.0

cm) and positioned at the bottom of the larger container with folded filter paper. The larger capsule was then capped and heat sealed. All capsules were washed in 10% $HNO_3$ and thoroughly rinsed in de-ionised $H_2O$. The sample capsules were handled with disposable plastic gloves. The samples were irradiated (8—15 min) in the In-Core-Irradiation System (ICIS) facility at the University of London Reactor Centre at Silwood Park, Ascot, U.K. The ICIS facility permitted rapid placement and transfer of samples into the thermal neutron beam by a pneumatic system (26 lbs/in$^2$, $N_2$). The thermal neutron flux was $1.5 \cdot 10^{12}$ n $\cdot$ cm$^{-2}$ $\cdot$ s$^{-1}$ at a reactor power of 100 kW. The prominent reaction utilising thermal neutrons is the (n, $\gamma$) reaction depicted as follows:

$^{A}_{Z}X + {}^{1}_{0}n \rightarrow [{}^{(A+1)}_{Z}X]^* \rightarrow$ particle and electromagnetic radiation (gamma rays)

X is the nuclide of interest, $A$ is the atomic mass unit, $Z$ is the atomic number, n is the neutron and * denotes an excited or unstable state which decays by the release of particles and gamma rays. Gamma ray spectrometry was accomplished by the use of a germanium-lithium (GeLi) detector (200 mm$^2$ X 5 mm) cooled with liquid nitrogen, coupled to an ADC Northern Scientific NS62318192 multichannel analyzer (4096 channels, 1 kev per channel) and a Nova Computer system. The output was displayed on a 9 inch Shibaden Videoscreen from which the peaks (gamma ray energies) could be chosen for analysis.

Chlorophyll fluorescence measurements were made as previously described [37] and chlorophyll levels were obtained by the method of Arnon [8].

## Results

*Ionic levels associated with the thylakoid membrane*

Throughout the work presented in this paper, ionic analyses has been restricted to determining levels of $K^+$, $Na^+$, $Mg^{2+}$, $Ca^{2+}$ and $Cl^-$. These species are the major osmotically active inorganic ions in biological tissue and moreover they are all detectable as radioisotopes after short irradiation times in a suitable neutron beam. The level of Mn has also been recorded in some cases since it is conveniently measured by neutron activation and also is an important element in the photosynthetic membrane.

The rationale of the experiments conducted with isolated thylakoids was to determine the level of ions associated with this membrane system after subjecting them to various ionic pretreatments. In many experiments $Na^+$ and $Mg^{2+}$ are used as the monovalent and divalent cations respectively. The reason for this is that these cations were detected with the greatest accuracy but as will be shown later, similar results could be obtained when $K^+$ and $Ca^{2+}$ levels were followed.

Fig. 1a shows the results of treating isolated thylakoids with various levels of $Mg^{2+}$ but keeping a constant background level of 10 mM $Na^+$. Both cations were added as their chlorides. In this experiment the membranes were initially treated with a large excess of monovalent cations (100 mM NaCl) in order to exchange other ions which may be at the membrane surface with $Na^+$. After this they were washed and resuspended in the experimental medium containing 10 mM NaCl as described in the Methods. The data shown indicates the ease by which $Mg^{2+}$ can displace $Na^{2+}$ associated with the membranes. For example,

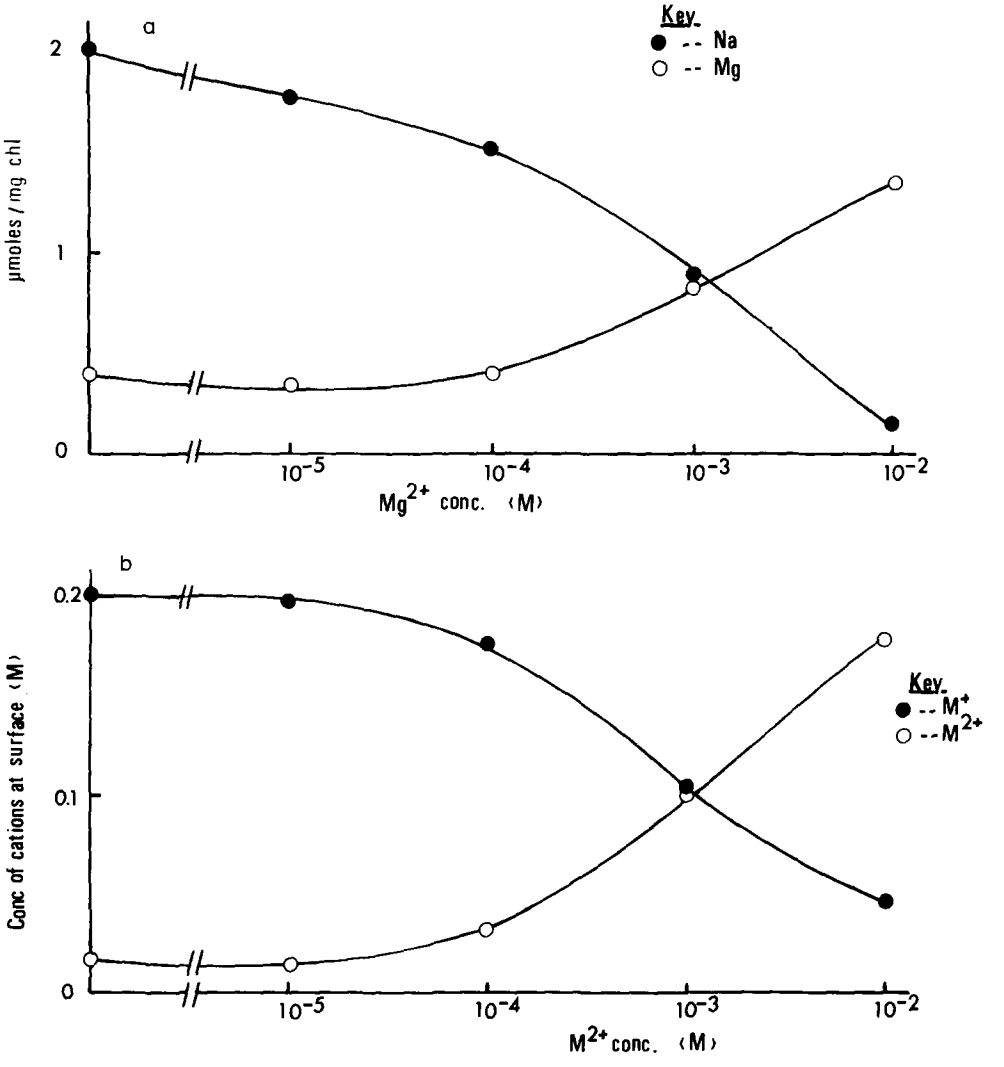


Fig. 1. (a) Neutron activation analysis of isolated thylakoid membranes equilibrated in solutions containing a constant background level of 10 mM NaCl but various concentrations of $MgCl_2$. Treatment and analyses carried out as in Methods. (b) Theoretical curves calculated using Eqns. 2 and 3 showing displacement of monovalent cations from a negatively charged surface by divalent cations for conditions comparable to the experiment in Fig. 1a. That is, $C'_\alpha$ = 10 mM and $C''_\alpha$ varied from 0 to $10^{-2}$ M. The calculations assume symmetrical electrolytes and the surface charge density ($\sigma$) was taken to be $-2.5$ $\mu$Coul/cm$^2$.

approximately half of the membrane associated $Na^+$ was displaced when the bulk monovalent/divalent cation concentration ratio was 10.

The ability of divalent cations at low concentrations effectively to displace monovalent cations at membrane surface is readily explainable assuming only electrical parameters are important and specific chemical interactions do not occur. One approach to estimate concentrations of ions at the surface of charged membranes is to combine the Gouy-Chapman theory and the Boltzmann equation [31,33].

The Gouy-Chapman theory leads to an expression which relates the membrane surface potential ($\psi_0$) to the surface charge density ($\sigma$) and the electrolyte concentration of the bulk solution $C_\alpha$. For a symmetrical charged electrolyte

$$\sigma = \pm\left[\frac{RT\epsilon}{2\pi}\sum_i C_{i\alpha}\left(\exp\frac{-Z_iF\psi_0}{RT} - 1\right)\right]^{1/2} \quad (1)$$

where $F$ is the Faraday, $R$ is the gas constant, $\epsilon$ is the permittivity of water and $T$ is the absolute temperature. Given a value of $\sigma$ this equation can be used to calculate $\psi_0$ for any particular mixture of electrolytes. For the experiments presented in this paper the bathing medium contains a mixture of monovalent electrolyte of concentration $C'_\alpha$ and divalent electrolyte of concentration $C''_\alpha$. Thus Eqn. 1 yields the quadratic:

$$2C''_\alpha \cosh^2\left(\frac{F\psi_0}{RT}\right) + C'_\alpha \cosh\left(\frac{F\psi_0}{RT}\right) - \left(2C''_\alpha + C'_\alpha + \frac{q^2}{2A^2}\right) = 0 \quad (2)$$

where $A = \left(\frac{RT\epsilon}{2\pi}\right)^{1/2}$

knowing $\psi_0$ and applying the Boltzmann expression:

$$C_0 = C_\alpha \exp\left(\frac{-ZF\psi_0}{RT}\right) \quad (3)$$

the surface monovalent ($C'_0$) and divalent ($C''_0$) cation concentration can be calculated.

Using Eqns. 2 and 3 values of $C'_0$ and $C''_0$ have been calculated for the conditions used in the experiment of Fig. 1a that is $C'_\alpha$ = 10 mM and $C''_\alpha$ varying from zero to $10^{-2}$ M. (Note that $MgCl_2$ was used in the experiment while the theory assumes that the anion carried two negative charges; however, in practice this will have very little effect on the calculation since the membrane surface is negatively charged). A value of $\sigma = -2.5$ $\mu$coulombs/cm$^2$ has been used since a number of different approaches give this to be a reasonable estimate for the surface charge density of the thylakoid membranes [27,32,36]. The outcome of the calculations are shown in Fig. 1b. Bearing in mind the assumptions of the Gouy-Chapman theory (see ref. 31) and that the concentrations are calculated only for the very surface plane of the diffuse layer the theoretical curves match the experimental data remarkably well in terms of predicting the concentration of divalent cations to bring about 50% removal of surface monovalent cations.

However when the reciprocal experiment was done, that is following the displacement of divalent cations from the membranes by adding various levels of monovalent cations, the theoretical and experimental curves did not match so closely (cf. Fig. 2a with Fig. 2b). Fig. 2a shows the results of analysing thylakoids treated with increasing levels of NaCl (0—$10^{-1}$ M). The membranes had been initially treated with 20 mM $MgCl_2$ to exchange surface cations for $Mg^{2+}$ and then after washing, suspended in the experimental medium containing 1 mM $Mg^{2+}$. When compared with the theoretical curves calculated using equations 2 and 3, then it seemed that in practise a much higher concentration

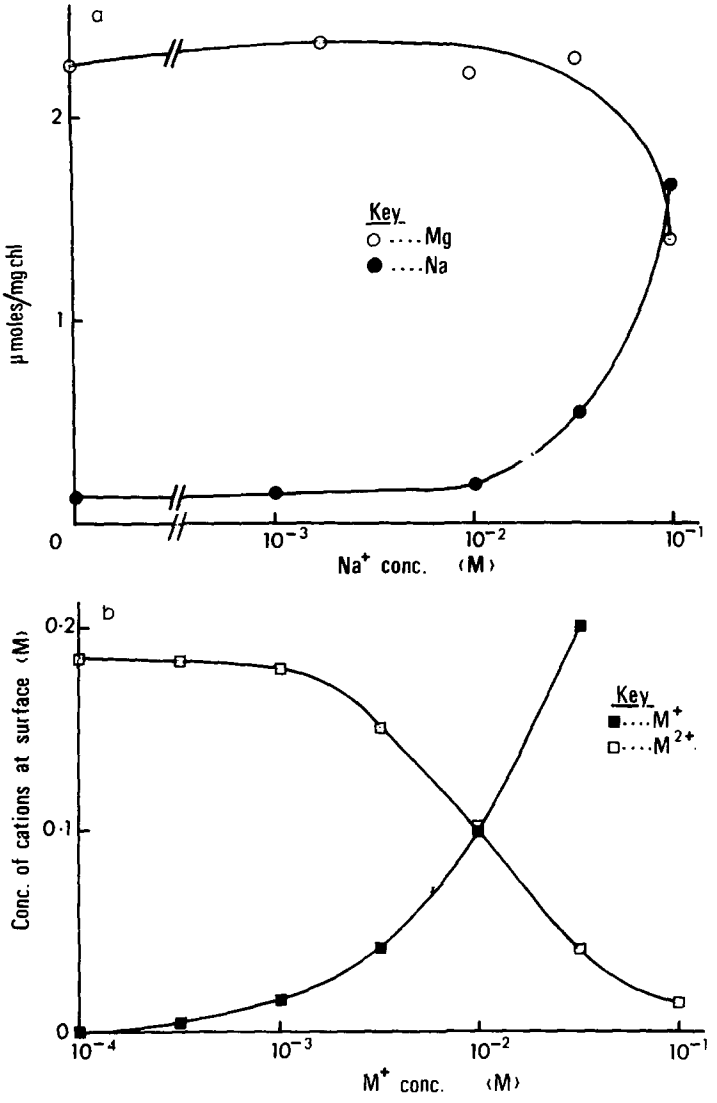


Fig. 2. (a) Neutron activation analysis of isolated thylakoid membranes equilibrated in solution containing a constant background level of 1 mM $MgCl_2$ but various concentrations of NaCl. (b) Theoretical curves calculated using Eqns. 2 and 3 showing displacement of divalent cations from a negatively charged surface by monovalent cations for conditions comparable to the experiment in Fig. 2a, that is, $C''_\alpha = 1$ mM and $C'_\alpha$ varied from 0 to $10^{-1}$ M. Other conditions are the same as Fig. 1.

of $Na^+$ was required to replace the membrane associated $Mg^{2+}$ than was anticipated.

In addition to using $Na^+$ and $Mg^{2+}$, experiments were conducted with $K^+$ and $Ca^{2+}$ as typical physiologically important mono- and di- valent cations. The results are shown in Table I. The thylakoids were initially treated with a high concentration of monovalent cations (100 mM $Na^+$), washed and then treated with either 20 mM divalent cations or 100 mM monovalent cations. The results of the subsequent analyses varied to some extent for different thylakoid prepa-

TABLE I

CATION EXCHANGE PROPERTIES OF THYLAKOID MEMBRANES

The membranes were initially treated with 100 mM NaCl for 15 min, washed twice with 0.11 m sorbitol/Tris, pH 7.6, and divided into samples for treatment with either 20 mM divalent or 100 mM monovalent cations. After 10 min treatment the thylakoids were washed twice with above sorbitol/Tris buffer.

| Element analysed (μmol/mg Chl.) (±SEM of 4 samples) | Treatments | | | |
|---|---|---|---|---|
| | 20 mM $Mg^{2+}$ | 20 mM $Ca^{2+}$ | 100 mM $Na^{+}$ | 100 mM $K^{+}$ |
| Mg | 1.040 ± 0.057 | 0.340 ± 0.095 | 0.32 ± 0.070 | 0.246 ± 0.046 |
| Na | 0.027 ± 0.002 | 0.023 ± 0.082 | 2.067 ± 0.263 | 0.060 ± 0.005 |
| K | 0.207 ± 0.035 * | 0.014 ± 0.004 | — | 2.364 ± 0.278 |
| Ca | 0.050 ± 0.007 | 1.296 ± 0.052 | 0.198 ± 0.022 | 0.122 ± 0.015 |

* This $K^{+}$ level was unusually high compared with the other comparable sets and with results of other experiments suggesting some contamination error in the analyses.

rations but the initial 100 mM NaCl pretreatment seemed to help minimise variability. Overall, the data indicated that exchangeability of the various cations tested is controlled by their charges rather than their chemical nature.

*Relationship between surface cations and chlorophyll fluorescence*

Recently it has been argued that cation-induced changes of chlorophyll fluorescence is controlled by cation exchanges in the diffuse layer adjacent to the thylakoid surface [31,32]. When thylakoid membranes are carefully isolated and suspended in a cation free medium the yield of chlorophyll fluorescence is at a maximum. Addition of 5 to 10 mM monovalent cations decreases the yield to a lower value but further additions of monovalent (100 mM) or low concentrations of divalent cations (5—10 mM) restores the fluorescence to the original high level [14,32—39]. These changes are independent of the redox state of the photosystem two trap since they are done in the presence of DCMU. To explain these effects Barber et al. [31—33] have argued that in the cation free medium the thylakoids retain a high level of divalent cations ($Mg^{2+}$) carried over during their isolation and that the subsequent fluorescence decrease results from the removal of this cation from the surface on the introduction of monovalent cations to the medium. The effect of this cation exchange in the diffuse layer is to reduce the positive space charge density immediately adjacent to the membrane surface which can then be restored to its original value by either adding back low levels of divalent cations or high levels of monovalent cations (see refs. 31—33).

To demonstrate these postulated cation exchanges, neutron activation analyses and chlorophyll fluorescence measurements were carried out on similar thylakoid samples. The results are shown in Fig. 3 where it can be seen that when thylakoids are suspended in a cation free medium so that the chlorophyll fluorescence is a maximum, $Mg^{2+}$ is the major membrane associated cation with a lower level of $Ca^{2+}$. The figure also shows that this $Mg^{2+}$ is removed from the membrane on adding NaCl to the suspension medium and at the same time the fluorescence went through its characteristic minimum [14,32,39]. 10 mM $Mg^{2+}$ added upon 10 mM $Na^{+}$ also restored the chlorophyll fluorescence to a maximum (not shown).

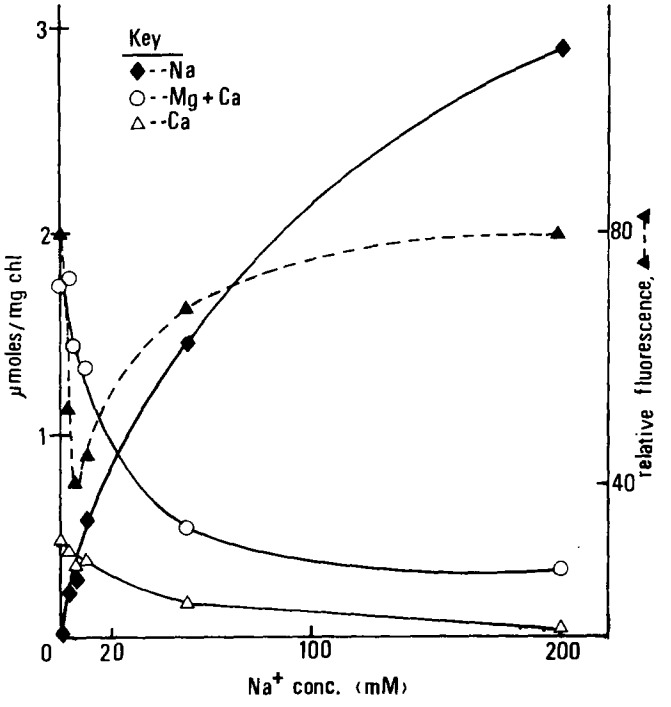


Fig. 3. Neutron activation analysis (———) and chlorophyll yield measurements (- - - - -) of thylakoids treated with various levels of NaCl. Thylakoids were obtained by subjecting isolated intact chloroplasts to hypotonic shock followed by suspension in 0.11 M sorbitol brought to pH 7.6 with Tris. 10 μM DCMU was added to the suspension. Chl concentration was approx. 20 μg/ml for the fluorescence measurements and 100 μg/ml for neutron activation analysis. After NaCl addition the thylakoids were washed twice with the sorbitol/Tris medium. The $K^+$ level upon hypotonic shock averaged less than 0.1 μmol/mg chl.

*Ionic content of intact chloroplasts*

Above it has been demonstrated that the cation species associated with the negatively charged thylakoid membrane is determined by the ionic nature of the medium in which they are suspended. It has also been clearly shown that divalent cations are preferentially attracted to the surface and indeed the experiment shown in Fig. 3 demonstrates that when thylakoids are carefully isolated

TABLE II

IONIC ANALYSIS OF INTACT PEA CHLOROPLASTS BY NEUTRON ACTIVATION

The mean intactness determined by the ferricyanide test [36] for the preparations used was 91%. Washing and suspension medium was 0.33 M sorbitol brought to pH 7.6 with Tris base.

| Element | μmol/mg Chl (S.E.M. of 17 sets) |
|---|---|
| Na | 0.22 ± 0.02 |
| K | 2.56 ± 0.06 |
| Mg | 1.72 ± 0.04 |
| Ca | 0.76 ± 0.03 |
| Cl | 0.21 ± 0.01 |
| Mn | 0.017 ± 0.0003 |

so that they only come into contact with cation free medium, the major cation associated with them is $Mg^{2+}$. Thus the question arises as to the nature of the ionic content of the physiological medium which normally surrounds the thylakoids in the intact organelle (i.e. stroma). We have carried out detailed neutron activation analyses of isolated chloroplasts retaining their outer membranes and ability to fix $CO_2$ (see Table II). Although the major intrachloroplast cation seems to be $K^+$, the $Mg^{2+}$ level (excluding the chlorophyll component) is only slightly less.

## Discussion

Because at pH values above 5 the thylakoid membrane carries a net negative charge [30], cations are attracted to its surface. As long as the association is electrical then the Gouy-Chapman theory predicts that the ions in the diffuse layer adjacent to the membrane surface will be determined by the ionic composition of the suspending medium. Of particular importance is the fact that under some conditions the ionic levels in the diffuse layer may be quite different from the levels in the bulk medium with divalent cations preferentially attracted to the surface relative to monovalent cations. The ionic analyses reported in this paper bear out this concept. It would be unreasonable to expect a very close quantitative agreement between the theoretical calculation and the experimental findings. The calculations have been done only for the concentration of ions immediately adjacent to the membrane rather than for the complete diffuse layer. Moreover there are many assumptions implicit in the Gouy-Chapman theory including the treatment of ions as point charges and the neglect of activity coefficients (see ref.31). On the experimental side, washing of thylakoids after their various pretreatments but before their analyses, almost certainly will remove some of the ions associated with the diffuse layer. In addition there may also be some specific chemical binding of cations to the membrane surface. Nevertheless bearing in mind these points, the ionic analyses of thylakoids pretreated with various ionic solutions do enforce the basic ideas emphasised in this and previous papers [26,30—33,34]. Particle electrophoresis studies have clearly shown that divalent cations screen the surface charges more effectively than monovalent cations and also demonstrated the lack of specificity of inorganic cations carrying the same charge [30]. The same dependence on the charge carried by the cation rather than its chemical nature in bringing about effects at the membrane surface has also been demonstrated by studying the fluorescence of both in vivo chlorophyll [32,33] and 9-amino acridine [41,42]. We have shown in this paper that the salt induced changes in chlorophyll fluorescence are associated with cation exchanges taking place at the membrane surface in a way predicted in earlier publications [31—33]. A point worth noting is that a higher concentration of monovalent cations was required to displace the surface divalent cations than predicted from the theory (compare Figs. 2a and 2b). A similar effect has also been seen when comparing experimental and theoretical data for electrophoretic measurements [30]. The reason for this is not clear but probably reflects the inadaquacy of the equations used to allow for important physical properties such as hydration energies. Nevertheless this finding further emphasise the preference

of the surface negative charges to be neutralized by divalent rather than monovalent cations.

An important outcome of the direct elemental measurements and the double layer concepts presented in this and earlier papers is that it allows for a better understanding of ionic regulation in the intact chloroplast. We have shown that the major cations in the isolated intact chloroplasts are $K^+$ and $Mg^{2+}$ and that the $K^+/Mg^{2+}$ ratio is small. Thus as long as the stromal activities of these cations are not high then it would be predicted that $Mg^{2+}$ is the major cation at the in vivo thylakoid membrane surface and that $K^+$ is mainly in the stromal phase. This has already been directly indicated by a number of observations [7,30,32, 49] and its consequence discussed in some depth [43]. In this present paper we report direct evidence that this is true, since analyses of isolated thylakoids which had not been subjected to any ionic washes, and maintained a high level of chlorophyll fluorescence, had mainly $Mg^{2+}$ and not $K^+$ associated with them (see Fig. 3).

The realization that the ionic distribution in the intact chloroplast is governed by the extensive negatively charged thylakoid membrane surface has important implications and explains a number of earlier observations. Some years ago Hind et al. [18] had shown that the counterion for the $H^+$-pump of the thylakoids was dependent on the ionic nature of the suspending medium. Only when the $K^+/Mg^{2+}$ ratio was high did $K^+$ act as the major counterion. With low $K^+/Mg^{2+}$ ratios, $Mg^{2+}$ acted as the counterion to the proton pump. This is understandable if the $H^+$ taken up exchanges with the cations at the membrane surface. Thus it would be expected that in the intact organelle proton pumping would exchange with the cations on the inner thylakoid surface which are predicted to be $Mg^{2+}$. Indeed the recent work of Portis and Heldt [44] and Krause [45] have indicated that $Mg^{2+}$ does act as the major counterion for in vivo proton pumping mechanism and as such is important in regulating the activity of the $CO_2$ fixing enzymes [44,50]. The fact that $Mg^{2+}$ is probably the major cation at the thylakoid membrane surfaces in the intact organelle would also explain the differential effect of the ionophores, nigericin and A23187 on uncoupling electron flow [46,47] and inhibiting high energy quenching of chlorophyll fluorescence [48,49]. The lack of effect of nigericin observed in these studies with intact chloroplasts would therefore reflect the low concentrations of $K^+$ near the membrane surface restricting its uncoupling action. On the other hand A23187 which promotes a divalent cation-proton exchange would have a ready supply of $Mg^{2+}$ at the membrane surface. Experiments with isolated thylakoids suspended in media containing various $K^+/Mg^{2+}$ ratios demonstrate this point (Barber, unpublished).

Throughout this paper we have avoided quoting ionic levels in terms of normal concentration units, that is, in terms of stromal and intrathylakoid volumes. In our opinion such units are meaningless bearing in mind the expected heterogeneity of the ionic distribution in an organelle where an extensive charged surface area of membrane is a major feature. Moreover, the effect of this large charged surface area together with the high protein content of the stroma almost certainly suggests that the thermodynamic activities of the ions will be much lower than their concentrations.

Finally it may be asked why $Mg^{2+}$ seems to be the physiologically important

cation in the intact chloroplast especially since the thylakoid membranes show no obvious chemical specificity for a particular cation. The answer may lie in the fact that significant quantities of $Mg^{2+}$ are required in the chloroplast for chlorophyll synthesis and there is an in vivo mechanism for maintaining a ready supply of this element to the organelle.

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